

# GENETIC EVENTS IN COLORECTAL TUMORIGENESIS

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Submitted for the degree of Doctor of  
Philosophy

University of Edinburgh

1995



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## ACKNOWLEDGEMENTS

This project was funded by the Cancer Research Campaign and I am grateful to them for their financial support.

My supervisors for this project were Professor Andrew Wyllie and Dr Martin Hooper, and I would like to thank them both for their help, patience and enthusiasm.

I would also like to thank Professor Bird and all members of the Pathology Department who contributed to this thesis. In particular I would like to thank Susan White and Derek Bishop for immunocytochemical data, and Dr Colin Purdie and Dr Pauline Carder for additional p53 data. I am also grateful to Drs Jill Bubb and Sarah Gledhill for helpful discussion and Bob Morris for technical assistance.

FISH analysis of *APC* was performed at the Western General Hospital MRC Human Genetics Unit under the supervision of Judy Fantes, Shelagh Boyle and Susan Farrington. The SW480 cell line was kindly provided by Dr Chris Marshall (ICRF). I would also like to thank Dr Alan Clarke for helpful (!) comments on the *APC* targeting study.

I am grateful to Lucy Curtis for the SSCP analysis of *MCC* positive control samples (kindly provided by Dr Ken Kinzler), and additional sequencing data, also for helpful discussion along the way.

I would also like to extend my gratitude to Mr Malcolm Dunlop for providing DNA samples for *MCC* analysis.

Finally, on a more personal note, I would like to thank all who have helped to keep me the same calm, well balanced, sane and rational individual I was when I arrived in the Department, in no particular order, Ann, Lucy, Steve, Jude, Emma, Dave, Ian and last but not least Damian. I would also like to express my love and thanks to my family for their continued support both emotional and financial over the previous years, and I would also like to assure Dad that I am at last going to get a "proper job" and anyway his smile will make it all worthwhile!

**THE GENE WILL OUT!**

## **DECLARATION**

The work presented in this thesis has been carried out by myself except where specifically indicated in the acknowledgements.

K.J.Cripps June 1995.

## ABSTRACT

More is known about the genes involved in colorectal tumorigenesis than for any other human cancer. Mutations have been identified in many genes, including *K-ras*, *APC*, *MCC*, *DCC* and *p53*. However, whilst much is known about these events, there are many questions that remain unanswered. Three specific questions involving the *p53*, *MCC* and *APC* genes were addressed in this thesis.

Firstly, the relationship between *p53* mutation and stabilisation of p53 protein was assessed. It had been previously assumed that stabilised p53 protein product was indicative of point mutation within the *p53* gene. More recently, however, situations have been described in which this relationship is not absolute. Accordingly, the incidence of stabilised product detected by immunocytochemistry (ICC) using p53 specific antibodies, was compared in a series of 47 colorectal carcinomas with that of mutation, detected by single strand conformational polymorphism analysis (SSCP) and sequencing of p53 exons 5-8. The results suggest a high correlation between the detection or absence of p53 protein stabilisation and the presence or absence of mutations within the gene (35/47:74%). However, specific incidences are highlighted in which concordance is not absolute. In particular, mutations in exon 6 of *p53* do not appear to result in stabilised protein and 2 tumours with a high degree of staining contained no apparent mutation within the entire coding region of the gene. Eight tumours within the series with positive ICC staining have also been shown to have no mutations within exons 4-9, a region where the majority of mutations of *p53* have previously been shown to occur. Possible explanations for the observed lack of an absolute concordance are discussed.

Secondly, mutations in *MCC* were studied in a subset of colorectal carcinomas. Although the *MCC* gene, on chromosome 5q21, was identified prior to *APC*, and

found to be mutated in some colorectal cancers (hence Mutated in Colorectal Cancer), its role in colorectal carcinogenesis is still uncertain. Only five sporadic colorectal tumours with *MCC* mutations have been published and no germline mutations of the gene have been identified in Familial Polyposis Coli (FPC) patients. A previous study from the Edinburgh Cancer Research Campaign Group had shown that whilst 5q21 allelic losses in a series of twenty colorectal carcinomas invariably involved *MCC*, no mutations of the remaining allele could be detected. It remained possible however, that *MCC* might be significant in other forms of colon cancer. Mutations were therefore sought in the coding region of *MCC* by SSCP in five colorectal tumours from non-polyposis patients who had presented with colon cancer at a young age (less than 48 years). Tumours had been maintained as xenografts in SCID mice and were therefore considered to be free from human contaminating (normal) stromal elements. No mutations were found in any of the young cases, although all previously published tumours with *MCC* mutations were detectable by the same analysis. These results therefore add to the evidence that inactivation of *MCC* by mutation is not important in colorectal carcinogenesis at least at the level detectable by this study.

Finally, essential preliminary steps were taken in establishing an experimental system in which to study the role of *APC* mutation in colorectal carcinogenesis. *APC*, which is close to *MCC* on 5q21, is mutated in at least 60% of sporadic colorectal tumours, and germline transmission of *APC* mutations are responsible for FPC. Mutations in *APC* are believed to be an early event in colorectal tumour progression and are detectable at equal frequency in benign and malignant tumours. It is of interest to establish whether such 'early' events are still important in determining the phenotype of tumours at later stages of progression, as this would influence gene therapy strategies in colorectal cancer. Accordingly, gene targeting constructs were designed

to execute a "Hit and run" strategy that would convert a known truncating mutation in the *APC* gene in the colorectal cancer cell line SW480 to wild type. The SW480 cell line was characterised by a variety of techniques and shown to contain three mutated copies of the *APC* gene, no normal copies and to be tumorigenic in SCID mice. In addition to a putative correcting construct (which remains to be confirmed and tested), a similar construct was made that would introduce a novel mutation 21 codons from the corrected one. This latter construct has provided a control for the efficiency of targeting the *APC* gene in this cell line.

# **CHAPTER 1**

## **INTRODUCTION**

### **1.1 The Pathology of Colorectal Neoplasia**

Neoplasms of the colon and rectum are very common. Malignant tumours of the large intestine are a frequent cause of human cancer mortality in the Western world with approximately 19,000 deaths in the UK per annum from colorectal cancer. Benign neoplasms may occur with a frequency of one person in three in the general population over the age of sixty (Cancer Research Campaign, 1993).

Cancer of the colorectum becomes increasingly common in older generations and the risk of a malignancy developing increases with age (Kozuka et al., 1975; Konishi et al., 1982). The idea that cancer may be a multistep process was proposed as long ago as 1949 and colorectal cancer provides an excellent model of this multistep nature (Berenblum and Shubik, 1949; Armatige and Doll, 1957; Foulds, 1958). Both benign and malignant neoplasms are relatively easy to obtain from surgical resection and colonoscopic polypectomy specimens, although often only after symptoms of carcinoma have been identified. The study of both benign and malignant tumours combined with evidence that colorectal cancer has both a genetic and environmental aspect can provide an analysis of both inherited and somatic gene changes and their relative roles in the multistep nature of colorectal cancer to be studied.

## 1.2 Colorectal Adenomas and Adenocarcinomas

Adenomas of the colorectum are benign tumours derived from the epithelium of the large intestine and are found relatively frequently throughout the population. It is thought that defects in stem cells near the base of the intestinal crypts may lead to adenoma formation as stem cells already have many of the characteristics of neoplasia e.g. they are immortal and undifferentiated. Increases in the number of stem cells without changes in the differentiation that occurs as cells migrate up the crypt may create the disturbance required for adenoma formation (Potten et al., 1984). Colorectal adenomas may be either polypoid or flat. Polypoid adenomas can be small or large, sessile or pedunculated. There is a range of histological types associated with polypoid adenomas of the colorectum presenting as a spectrum from tubular through tubulovillous to villous. Tubular forms are the most common and villous adenomas the least often found but the latter have the highest malignant potential (Morson, 1990; Clark et al., 1985; Muto, 1975). The cytological organisation within both polypoid and flat adenomas shows a wide spectrum of localised dysplasia, merging at its most severe with the diagnosis of *carcinoma in situ*. Small adenomas usually exhibit mild dysplasia and are evenly distributed throughout the colorectum, whilst larger adenomas have more severe dysplasia, a correspondingly higher malignant potential and tend to concentrate in the left colon and rectum, especially the sigmoid section. Flat adenomas are generally moderately dysplastic (Muto, 1975; Enterline et al., 1962; Konishi et al., 1982.). An increase in dysplasia has been linked with

increasing age, number and size of polyps, especially those with a villous type histology. Commonly, severely dysplastic adenomas are associated with frank adenocarcinoma in the same region of the colon, leading to the suggestion that dysplasia may be a useful marker for increased colorectal cancer risk (Kalus, 1972). The size and level of dysplasia as well as number of adenomas correlates with malignant potential (Muto et al., 1975). It should be noted that dysplasia is important in other clinical contexts in the colorectum such as ulcerative colitis, Crohn's disease (which has been shown to predispose to colorectal cancer at a younger than average age) and schistosomiasis (Morson, 1990; Hamilton, 1985 Ming-Chai et al., 1980;). There is evidence for the generation of malignant tumours from generalised dysplastic epithelium in patients with Ulcerative Colitis without an adenomatous stage suggesting *de novo* carcinoma can also arise (Hamilton 1992). In general, histology reveals a continuous spectrum of adenomas from small with mild dysplasia (most of which do not grow and may even regress) to large, highly dysplastic polyps with villous architecture and an increased malignant potential.

Several physical factors affect both occurrence and distribution of colorectal adenomas. Firstly, the distribution of adenomas appears to be age related. The left colon and rectum are the most common sites for adenomas in young patients, whilst the right colon is more common in older patients (Gerharz et al., 1987). In all areas of the colon, adenomas are more common in males, whilst at sites other than the colon the cancer bias is slightly towards females. Other studies show that adenomas in the



sigmoid colon and rectum are generally larger and more dysplastic in females (Hoff et al., 1985). Therefore age, sex and site are important variables in adenoma formation. Additionally, abnormal DNA content or aneuploidy is less common in adenomas than carcinomas and increases with increasing dysplasia indicating a poorer prognosis (Goh and Jass, 1986; Quirke et al., 1986; Enterline and Arvan, 1967; Armitage et al., 1985). The majority of cancers of the colorectum are adenocarcinomas although rarer types also exist (Jass and Morson, 1987; Morson, 1974). Multiple neoplasms both benign and malignant are common in the colon and rectum, and synchronous and metachronous carcinomas are more common in cases with synchronous adenoma.

The literature remains divided on the true origins of colorectal carcinomas and it has been proposed that carcinomas may arise both from within existing adenomas, in what has been termed the adenoma to carcinoma sequence (Muto et al., 1975), or via areas of generalised dysplasia (*de novo*) without an adenomatous stage. Whilst it is probable that some colorectal cancers originate in adenomas, the majority of adenomas do not appear to progress to carcinoma and indeed may even regress (Knoernschild, 1963).

### **1.3 Evidence for the Adenoma to Carcinoma sequence**

Many pieces of evidence, both temporal and spatial, support the adenoma to carcinoma sequence. Firstly, it has been suggested that carcinomas develop from within adenomatous tissue (Bussey and Morson, 1975; Eide, 1983) and that as the carcinoma expands it destroys the surrounding adenomatous tissue (Morson, 1974).

Another study has stated that some colorectal cancers have residual adenomatous tissue at their periphery (Muto et al., 1975). Secondly, adenomas arise at an earlier age than carcinomas and are more common in carcinoma associated than non-carcinoma associated bowel-tissue (Eide et al., 1986). Thirdly, removal of adenomas decreases the incidence of associated carcinoma of the colon or rectum (Gilbertson and Nelms, 1978). Finally, carcinomas are clonal (Fearon et al., 1987), carrying the same genetic lesions (usually with additional lesions) as associated adenomas. This will be discussed in detail later. These data strongly suggest that at least some carcinomas arise from within existing adenomas.

#### **1.4 Environmental and Dietary Factors influencing Colorectal Cancer**

Environmental factors such as diet and geographical location have been implicated in colorectal and other tumour development (Doll and Peto, 1981; Bussey, 1990). Variation in the frequency of colorectal cancer has been noted over geographical areas. For example, cancer of the colorectum is common in North America and Europe, rare in Asia and uncommon in Africa (Boyle et al., 1985). This has been linked to environmental factors rather than racial features. Migrant studies on Japanese immigrants to the United States show that colorectal cancer, until recently uncommon in Japan, becomes an increasing problem as lifestyles adapt to the Western way of living (Haenszel and Kurihara, 1967). It has been suggested that animal fat in the diet may play a role in colorectal cancer development whilst cereal fibre intake,

vitamins C, D and E and calcium may show a negative correlation with colorectal cancer as well as cancer of the breast, prostate and ovary (Jain et al., 1980; Rose et al., 1986; Garland 1985; DeCosse et al., 1989; Newmark et al., 1984). However, studies on Maori populations who eat a high fat diet but have a low incidence of colorectal cancer suggest other factors are also involved (Smith et al., 1985). The specific dietary factors involved have not as yet been identified (Bruce, 1987). However a mutagenic lipid compound (faecapentaene) causes mutation, including single strand breaks and sister chromatid exchanges, in human cell lines and has been isolated from the faeces of people in high risk areas for colorectal cancer (Plummer et al., 1986). Faecapentaene is produced by the action of anaerobic bacteria and its synthesis is stimulated by an increase in concentration of bile acids (Gupta et al., 1984). Animal fat in the diet may increase faecal pH thereby promoting the formation of bile acids such as deoxycholic acid and lithocholic acid which may cause small adenomas to progress to larger adenomas (Hill, 1975; van der Werf et al., 1983). Deoxycholic and lithocholic acids are co-mutagens and increase the number of mutations in DNA over the mutagen alone (Wilpart et al., 1983). In animal models, bile salts and dietary fat also increase colonic ornithine decarboxylase activity which is involved in polyamine synthesis and is required for cell proliferation (Takano et al., 1984, Rozhin et al., 1984). Inhibition of ornithine decarboxylase by difluoromethylornithine has been shown to prevent chemically induced colonic tumours developing in mice (Kingsnorth et al., 1983). Adenomas and carcinomas have about 3 times the ornithine decarboxylase and 2 times the polyamine levels seen in

normal colonic mucosa and ornithine decarboxylase activity is higher in the normal tissue from colorectal cancer patients over non-affected individuals (LaMuraglia et al., 1986). Additionally, bile salts may activate protein kinase C (PKC) which is a regulator of cellular differentiation and growth (Craven et al., 1987). PKC activity is increased in colorectal cancers, compared to normal colon and also in patients with adenomas (McGarrity and Peiffer, 1994). However, case control studies of matched cancer and normal patients often show no direct link with fat, bile acids etc. This, and the recognition of families in which colon cancer is common (although families may share common diets and environment), suggest that genetic elements may be involved in colorectal cancer development.

## **1.5 The Genetic Basis for Colorectal Cancer**

Whilst evidence on environment, diet, age and sex suggest that these are risk factors for colorectal cancer, the lack of confirmation of involvement of these factors in all cases may suggest an underlying genetic basis for cancer formation. The majority of colorectal cancers are not associated with clear inherited syndromes although hereditary forms do exist, including Familial Polyposis Coli (FPC), Gardner's Syndrome, Hereditary non-Polyposis Colorectal Cancer (HNPCC) and Turcot's Syndrome. Other gastrointestinal syndromes such as Peutz-Jeghers disease and familial juvenile polyposis have only a slightly increased risk of colon carcinoma development compared to the normal population and will not be discussed.

The study of families suggested that colorectal cancer often occurs in families without a clear form of inheritance. Close relatives of patients with cancer were shown to develop cancer themselves at a higher frequency than the general population (Lovett, 1976). More recently a study has estimated that first degree relatives of colorectal cancer patients have a 2-3 fold increase in risk of developing the disease themselves (Burt et al., 1985). However, in this study, when cancer cases alone were considered, no clear Mendelian inheritance pattern existed. Family members, however, had a greater number of adenomas than control populations and when cancers and adenomas were analysed together an autosomal dominant inheritance of susceptibility to adenoma formation emerged. Large numbers of kindreds exist either with single adenomas or family clusters of colon cancer. This suggests that genetic factors may determine susceptibility to either cancer or adenoma formation, whilst additional genetic or environmental factors determine which susceptible individuals become affected (Cannon-Albright et al., 1988). The epithelial cell proliferative compartment shows a shift towards the luminal surface of colonic crypts in subjects with a strong family history and this abnormal proliferation is also seen in both FPC and HNPCC (Gerdes et al., 1993). Abnormal proliferation may also be seen in surrounding normal colorectal mucosa preceding neoplastic changes (Risio et al., 1991).

### **1.51 Familial Polyposis Coli (FPC).**

FPC has several characteristic features, including the appearance at a young age (usually in the late teens/twenties) of numerous (100's to 1000's) adenomatous polyps

lining the colorectal epithelium, an increased incidence of associated carcinomas (also at a correspondingly early age), and a known genetic origin (Bussey, 1990). Adenomas are usually distributed evenly around the colorectum or tend to the left (distal) side and most patients will develop randomly sited carcinomas if a colectomy (removal of the colon) is not performed (Lynch et al., 1991). A genetic element has been established by the study of many family histories, the first of which was reported in a brother and sister in 1882 (Cripps, 1882). FPC is inherited in an autosomal dominant fashion with a high penetrance and is a predisposition to adenoma formation not carcinoma *per se*. There is much reported variability within families in terms of age of onset, number and size of polyps and severity of disease. Extracolonic manifestations are also associated with FPC including desmoid tumours, thyroid, periampullary or duodenal cancer and hepatoblastoma (Jagelman, 1987; Iwama et al., 1993; Garber et al., 1988). Polyposis accounts for only a small percentage (typically <1%) annually of colorectal cancers, but it provides a good model in which to study the progression of tumours and provides a framework in which to study the more common sporadic tumours. FPC allows the study of small adenomas usually not detected in patients with sporadic tumours until carcinoma symptoms are present. A locus linked to FPC was mapped to chromosome 5q21 by the study of a patient with both polyposis and mental retardation (Bodmer et al., 1987; Leppert et al., 1987). The genetic elements of FPC will be discussed later.

### **1.52 Gardner's Syndrome.**

This syndrome applies to FPC families with additional extracolonic features such as multiple osteomas in all parts of the skeleton, multiple epidermoid cysts, fibromas of the skin, dental abnormalities and desmoid tumours of the abdomen and abdominal wall (Gardner 1951; Gardner and Richards, 1953). An additional feature is abnormal retinal pigmentation termed CHRPE (congenital hypertrophy of the retinal pigment epithelium) (Blair and Trempe, 1980). These are ocular lesions varying in size, configuration and distribution (Traboulsi et al., 1990). Osteomas are generally benign, and Gardner's Syndrome (GS) associated desmoid tumours do not metastasise. However, complications from desmoid tumours are the second most common cause of death behind metastasis of the primary tumour in FPC (Boland et al., 1989). Previous abdominal surgery seems to predispose to desmoid tumour formation (Gurbuz et al., 1994). Gardner's syndrome has been linked to chromosome 5q21, the same locus as FPC (Herrera et al., 1986).

### **1.53 Hereditary Non-Polyposis Colorectal Cancer (HNPCC).**

HNPCC accounts for 4-13% of colorectal cancers in Industrial countries. Unlike both FPC and Gardner's Syndrome multiple adenomas do not cover the entire colon, but small clusters or isolated adenomas and carcinomas occur, particularly in the right (proximal) and transverse colon (Lynch et al., 1993a). HNPCC is classified as follows: Families in which at least 3 relatives in 2 generations, one of which must be a first degree relative of the other 2, are affected, with at least one diagnosis of colorectal

cancer made at less than 50 years (Vasen et al., 1991). Tumours at other sites are common in HNPCC patients, including the endometrium, stomach, and biliopancreatic and urinary tracts (Mecklin and Jarvinen, 1991).

### **1.54 Turcots's Syndrome.**

This disease involves multiple adenomas of the colon in combination with neuroepithelial central nervous system tumours (Turcot et al., 1959). The most common tumour is glioblastoma. It has been postulated that Turcot's Syndrome is a pleiotropic, incompletely penetrant allelic variant of FPC and Gardner's syndrome with linkage to the FPC locus (Lewis et al., 1983; Lasser et al., 1994).

### **1.55 Hereditary Flat Adenoma Syndrome.**

This is an autosomal dominantly inherited predisposition to multiple colonic adenomas, although usually less than 100 in number. Adenomas are generally located in the proximal colon and are flat not polypoid. There is a strong association with cancer which tends to be mainly right sided and a median-to-late age of onset of adenoma formation of about 55 years (Lynch et al., 1991). This syndrome is also linked to the 5q21 FPC locus and presents similar upper gastrointestinal tract abnormalities to FPC. For example, cancer of the small intestine and fundic gland polyps of the stomach are commonly associated with Flat Adenoma Syndrome (Lynch et al., 1993b)



In summary, many studies of colorectal cancer have revealed multiple variables in cancer formation. There is evidence for an adenoma to carcinoma sequence, although it is probable that most adenomas never progress to carcinoma. There is also evidence for colorectal carcinoma development without a clear adenomatous precursor lesion. Finally, much evidence exists to suggest both an environmental and genetic aspect to colorectal cancer and hereditary forms of colon cancer have been described.

## Chapter 2

### The Genetic Changes Associated with Colorectal Cancer.

A widely held belief is that neoplasia is a result of activation of oncogenes, coupled with the inactivation of tumour suppressor genes.

Mutations in oncogenes tend to generate dominant growth promoting phenotypes. In contrast tumour suppressor genes usually follow Knudson's two hit model for inactivation with concomitant loss of function in a recessive manner (Knudson, 1985). Studies of allelic deletions in tumours have often suggested the location of putative tumour suppressor genes. One such study applied restriction fragment length polymorphisms (RFLP) analysis to study allelic losses in a series of colorectal cancers in what was termed an allelotype (Vogelstein et al., 1989). 75% of the tumours showed loss of 17p and 18q. 25-50% showed losses in 1q, 4p, 5q, 6p, 6q, 8p, 9q, 18p and 22q. In most deletions the loss occurred in only one of the 2 chromosome arms and therefore did not represent loss of the whole chromosome. Tumours with multiple deletions had a poorer prognosis than those with few deletions. Other studies showed loss of heterozygosity (LOH) by RFLP in both FPC and sporadic tumours on chromosomes 5, 14, 17, 18 and 22 (Sasaki et al., 1989; Monpezat et al., 1988). Several oncogenes and tumour suppressor genes have been shown to play a definite

role in colorectal tumorigenesis, whilst at other loci a correlation between LOH and colorectal cancer is less well defined.

## 2.1 The short arm of chromosome 17

Greater than 75% of colorectal carcinomas, but few adenomas show losses of 17p (Vogelstein et al., 1988). Loss of 17p is therefore generally considered to be an event occurring around the adenoma to carcinoma conversion (Nigro et al., 1989). Polymorphic 17p markers detected a common region of deletion in colorectal cancers localising to 17p13 (Baker et al., 1989), a region previously shown to contain the *p53* gene (van Tuinen et al., 1987).

### 2.11 The *p53* gene

*p53* was initially discovered due to its interaction with the SV40 large T antigen in transformed cells and, because large T antigen was required to maintain the transformed state, *p53* was termed a tumour antigen (Lane and Crawford, 1979). Subsequently *p53* was found in tumour and transformed cell lines whilst genomic and cDNA clones of *p53* were found to immortalise cells in culture and could co-operate with the *ras* oncogene to transform primary rat embryo fibroblasts. *p53* was therefore called an oncogene (Jenkins et al., 1984; Eliyahu et al., 1984; Parada et al., 1984). It was later discovered, however, that these transforming clones of *p53* were mutant (Hinds et al., 1989). However, wild type *p53* has been shown to suppress the transformation of cells by other oncogenes, the growth of transformed cells in culture,

and the tumorigenicity of cells in animals. Additionally, wild type *p53* alleles are commonly lost or mutated in human tumours (Finlay et al., 1989; Eliyahu et al., 1989; Baker et al., 1990a; Chen et al., 1990; Baker et al., 1989). These characteristics have led to the classification of *p53* as a tumour suppressor. Moreover, gain-of-function mutants of *p53* exist which can stimulate cell division even when in the heterozygous state (Halevy et al., 1990; Dittmer et al., 1993; Wolf et al., 1984; Chen et al., 1990).

The *p53* gene encodes a 393 amino acid nuclear phosphoprotein and several functional domains of the protein have been elucidated (Figure 1). An acidic N-terminus of the protein has transcriptional transactivational activity (Raycroft et al., 1990; Fields and Jang, 1990; Farmer et al., 1992), whilst a sequence specific DNA binding region located in the central core region of the protein has important functional significance (Kern et al., 1991; Bargonetti et al., 1993; Pavletich et al., 1993). *p53* binds to *p53* response elements in the DNA of target genes, and with nearby promoter sequences, stimulates expression of target genes in a *p53* dependent manner (Farmer et al., 1992). A consensus site for *p53* binding to DNA has been described (El-Diery et al., 1992). *p53* can also negatively regulate some genes that do not contain *p53* response elements including *RBI*, *PCNA* IL-6 and *p53* itself (Santhanam et al., 1991; Shiio et al., 1992). The N-terminal transcriptional activation domain of wild type, but not mutant *p53* can also bind to the TATA binding protein (TBP) which may suggest that *p53* functions by regulating genes involved in transcription either by direct interference with TBP or by blocking another factor from binding to TBP (Seto et al., 1992).

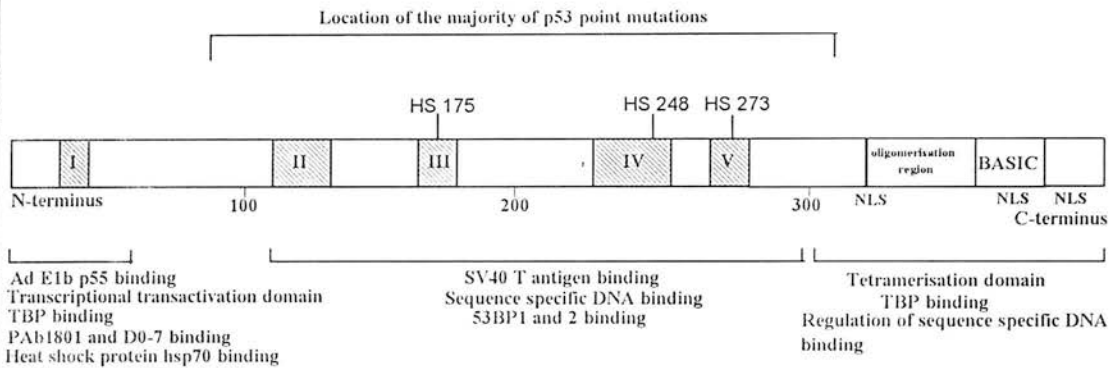
A non-sequence specific DNA binding region of *p53* is contained in the basic C-terminus of the protein, the sequence of which is predicted to form helical structures.

The C-terminus also contains regions necessary for nuclear localisation signals, single strand reannealing and protein tetramerisation (Shaulsky et al., 1990; Wang et al., 1993; Pavletich et al., 1993).

Oncoproteins of some transforming DNA viruses can bind to and inactivate wild type p53 function. These include the previously mentioned SV40 T antigen, the adenovirus E1B protein and the Papilloma virus type 16 and 18 E6 protein (Sarnow et al., 1982; Werness et al., 1990; Bargonetti et al., 1991; Mietz et al., 1992). Cellular oncoproteins can also bind to p53. These include the MDM2, TBP and RepA proteins (Momand et al., 1992; Olson et al., 1993; Seto et al., 1992; Dutta et al., 1993). Recent studies have also discovered two more cellular p53 binding proteins 53BP1 and 2, both of which bind to the central region of the p53 protein, which is also where SV40 T binds (Figure 1) (Iwabuchi et al., 1994). The half-life of mutant p53 can be altered by binding of p53 to the heat shock protein hsp70 (Finlay et al., 1988).

Wild type p53 is believed to be involved in monitoring DNA damage at various cell cycle checkpoints in a role that has been termed the "guardian of the genome" (Lane, 1992). The p53-dependent response to DNA damage can involve induction of a G1 arrest of the cell cycle possibly to allow DNA repair (Kuerbitz et al., 1992; Kastan et al., 1991). Primary murine fibroblasts that lack p53 are deficient in, or fail to activate a G1 checkpoint in response to ionising radiation (Kastan et al., 1992). Wild type p53 levels rise transiently in the skin after UV irradiation, possibly to allow the repair of specific UV induced mutations such as pyrimidine dimers (Hall et al., 1993).

**Figure 1. The structural domains of the p53 protein**



Structural domains on human p53 (based on Prives & Manfredi, 1993). The shaded boxes containing roman numerals represent the 5 conserved domains of p53. The three nuclear localisation signals (NLS) are also shown. The mutational hot spots are identified by amino acid number ( e.g. HS175). TBP is the TATA box binding protein.

Defects in this response to UV or ionising radiation can be seen in the cells of patients with the chromosome instability disorders of Ataxia Telangiectasia and Bloom's Syndrome (Kastan et al., 1992; Lu and Lane, 1993).

p53 can also induce apoptosis in some cells and this response may depend on the physiological state and type of the cell (Yonish-Rouach et al., 1993; Shaw et al., 1992; Slichenmyer et al., 1993). p53 has been shown to be essential for the apoptotic response to IR (ionising radiation) in murine thymocytes (Clarke et al., 1993; Lowe et al., 1993a.)

Mutations of *p53* have been detected in many types of human cancers including, lung, breast, liver, bladder colon and ovary and in chronic myelogenous leukaemia cells in blast crisis, usually at an advanced stage of neoplastic development (Nigro et al., 1989; Hollstein et al., 1991). In contrast, studies on some oesophageal and brain tumours have indicated that mutation of *p53* occurs earlier in tumour development at a pre-malignant stage (Bennett et al., 1991; Sidransky et al., 1992). The majority of mutations are point mutations and are generally missense mutations that produce a protein with an altered conformation, stability and biochemical properties such as loss of DNA binding activity and associated transcriptional activation (Gannon et al. 1990). Greater than 90% of the point mutations seen in tumours are clustered between amino acids 130-290 in the central core region of the protein and localised to one of four phylogenetically conserved regions between amino acids 117-142, 171-181, 234-258 and 270-286 (Figure 1) (Levine et al., 1991; Soussi et al., 1990). Moreover, there are at least three hotspots for mutation, at codons 175, 248 and 273. The majority of point mutations occur in the sequence specific DNA binding region of the gene suggesting that this region is fundamental to the growth suppressive effects of *p53*. The mutations themselves have different biochemical and biological properties which may be relevant in prognosis of patients with tumours (Raycroft et al., 1990; Fields and Jang, 1990). The frequency and distribution of mutations varies between tumour types perhaps reflecting differing cellular environments and cell specific selection pressures. The majority of mutations in both colon and gastric tumours are G-A transitions at CpG dinucleotides; in breast tumours by contrast, G-T

transversions are more common and rarely occur at CpG sites (Hollstein et al., 1991; Uchino et al., 1993). The majority of *p53* mutations are seen in tumours with a loss of 17p. Consequently, it has been proposed that point mutation of the gene is a rate limiting step and 17pLOH occurs after mutation, usually at the transition from benign to malignant neoplasm (Kikuchi-Yanoshita et al., 1992; Baker et al., 1990a; Shaw et al., 1991). Additionally, germline mutations of the *p53* gene are found between codons 245 and 258 in patients with the rare autosomal dominant Li-Fraumeni Syndrome in which affected individuals develop a spectrum of tumours at an early age (Li et al., 1988; Malkin et al., 1990; Srivastava et al., 1990).

It is of interest that the MDM2 oncogene is overexpressed in some sarcomas in which there is no *p53* mutation, and cervical cancers with HPV 16 and 18 also appear to contain no mutated *p53* (Oliner et al., 1992; Scheffner et al., 1990). This suggests that binding of cellular or viral oncoproteins is sufficient to destroy or abrogate wild type *p53* function without mutation of the *p53* gene itself. In the case of the HPV 16 and 18 E6 protein, this is in the form of degradation of p53 protein by the ubiquitin protease pathway (Scheffner et al., 1990). Mutant p53 protein complexes with the heat shock protein hsp70 in the cytoplasm (Stürzbecher et al., 1987) and it has been proposed that mutant protein may also complex with wild type protein thereby preventing wild type p53 from reaching the nucleus with concomitant loss of function, therefore representing a dominant loss of function mutation. Additional evidence for this includes the observation that a large amount of mutant protein is required to



transform cells in culture and similarly, experiments with temperature sensitive mutants of *p53* have shown that wild type protein protects cells from *ras* induced transformation whereas *p53* in the mutant conformation co-operates to transform cells (Hinds et al., 1989; Martinez et al., 1991). The relative amounts of wild type to mutant protein may be critical. Overexpression of wild type protein in cells also expressing mutant *p53* suppresses cell growth, transformation and tumorigenicity of the cells (Finlay et al., 1989; Baker et al., 1990b; Martinez et al., 1991). Additionally mutant *p53* has been shown to gain new functions in experiments where it was placed in cells with no endogenous wild type protein. This resulted in an increased ability of the cells to form tumours in animals (Wolf et al., 1984).

Further evidence for a critical role for *p53* in tumorigenesis is provided by murine models, in which mice have been created with no *p53* protein. These animals are developmentally normal but die of multiple tumours, particularly lymphomas, at an early age (Donehower et al., 1992). Heterozygous animals also develop tumours but the onset of development is delayed compared to the null animals (Harvey et al., 1993).

In summary, the *p53* gene has been shown to have growth suppressive properties and its mutated forms are frequently oncogenic. Wild-type *p53* has transcriptional transactivation and repression activity, can bind DNA either specifically or non-specifically, forms tetramers with itself and probably

functions in the control of DNA damage repair either by creating a G1 arrest or inducing apoptosis. Mutations in *p53*, especially in the conserved regions containing the sequence specific DNA binding domain, are common in many tumour types. These mutations are usually missense and abrogate sequence specific DNA binding and transcriptional transactivation of the protein, occurring commonly at the adenoma to carcinoma transition.

## 2.2 The long arm of chromosome 18

Deletions within the long arm of chromosome 18 occur in 75% of colorectal carcinomas, 47% of large adenomas and less than 10% of small adenomas in both sporadic and FPC patients (Cho and Vogelstein, 1992; Fearon and Vogelstein, 1990; Miyaki et al., 1990). Panels of polymorphic probes detected common regions of deletion to 18q21.3 and a gene, *DCC* (for deleted in colon cancer ), was identified (Fearon et al., 1989). It was shown to encode a membrane bound protein, the extracytoplasmic portion of which shows significant sequence homology to neural cell adhesion molecules and other related cell surface glycoproteins in the immunoglobulin superfamily. This is of interest as changes in cell surface interactions have been implicated in abnormal colonic differentiation (a characteristic of neoplasia). Additionally, tumour cells lose cell/cell and cell/basement membrane contacts and form inappropriate contacts during metastasis (Pignatelli and Bodmer, 1988). NIH3T3 cells expressing *DCC* can stimulate neurite outgrowth of rat PC12 cells when in cell/cell contact, thus replacing the endogenous neural cell adhesion

molecules (Pierceal et al., 1994). Relatively few somatic mutations of *DCC* have been described either in primary tumours, cell lines or xenografts, but those that have include point mutations in both introns and exons, a homozygous deletion of the 5' end of the gene and 10 cases of small insertions within an 170bp fragment immediately downstream of an exon (Fearon et al., 1990; Cho et al., 1994). *DCC* is expressed in most normal tissues but expression is greatly reduced in colorectal tumours, especially liver metastases, and loss of 18q correlates with a poorer prognosis (Itoh et al., 1993; Kikuchi-Yanoshita et al., 1992; Jen et al., 1994). A study of 318 primary tumours with no loss of 18q, revealed no amplifiable *DCC* mRNA, suggesting that epigenetic inactivation of the gene can occur as well as genetic inactivation (Iino et al., 1994). The addition of antisense *DCC* DNA has been reported to be tumorigenic in Rat-1 cells (Narayan et al., 1992). Additionally, microfusion of chromosome 18 to cancer cells decreases tumorigenicity (Tanaka et al., 1991; Goyette et al., 1992).

**In summary, the status of 18q correlates with prognosis and LOH of 18q is very common, occurring in about 75% of colorectal tumours, usually after malignant conversion. The gene *DCC* has sequence homology to neural cell adhesion molecules and although point mutations of the gene are relatively uncommon, decreased mRNA expression is commonly seen in colon tumours.**

## 2.3 The long arm of chromosome 5

Losses of the long arm of chromosome 5 occur in up to 60% of sporadic adenomas and carcinomas (Vogelstein et al., 1988; Solomon et al., 1987; Ashton-Rickart et al., 1989) and the gene for FPC has also been mapped to this region (Bodmer et al., 1987; Leppert et al., 1987; Meera-Khan et al., 1988). Two genes within 5q21, *MCC* and *APC* have been identified (Kinzler et al., 1991a; Joslyn et al., 1991; Kinzler et al., 1991b; Nishisho et al., 1991; Groden et al., 1991). Additionally, the tumorigenicity of colon cancer cells has been shown to be suppressed by the introduction of a normal chromosome 5 (Tanaka et al., 1991; Goyette et al., 1992). This provides evidence for the location of a tumour suppressor gene (or genes) within 5q21 which may be important in colorectal cancer.

### 2.31 The *MCC* gene

*MCC* was the first gene identified within the region of 5q21 commonly deleted in colorectal cancer by the discovery of a somatically rearranged restriction fragment in the DNA from a colon tumour (Kinzler et al., 1991a). The gene encodes an 829 amino acid protein which shows sequence similarity to the region of the m3 muscarinic acetylcholine receptor which specifies which G protein is involved in the acetylcholine stimulation of phospholipase C. This suggests that a possible function for *MCC* may be to bind and inhibit some G proteins thereby inhibiting signalling through the calcium dependent phosphoinositide pathway, a pathway that is mitogenic for some cells (Bourne et al., 1991a). Analysis of the *MCC* gene structure suggests a

discontinuous pattern of coiled coil domains separated by hinge regions like those seen in intermediate filaments, which may indicate homo- or hetero-dimerisation potential. The presence and distribution of heptad repeats within the gene suggest an ability to form  $\alpha$  helices perhaps in a kink-and-rod model (Bourne et al., 1991b). The *MCC* gene is expressed in both rodent and human colonic mucosa and disruptions to the gene have been identified in some colorectal tumours. Many studies have identified losses in 5q21 involving the region including and surrounding *MCC*. Allele losses of 5q have been identified in a variety of tumour types such as colon, lung, oesophagus, breast, kidney, liver and stomach (Miki et al., 1991; Ashton-Rickardt et al., 1989; D'Amico et al., 1992; Boynton et al., 1992; Thompson et al., 1993; Morita et al., 1991a and 1991b; Fujimoro et al., 1991; Sano et al., 1991). Losses of this region are also associated with dysplastic and cancerous ulcerative colitis (Greenwald et al., 1992).

A somatic rearrangement found within 5q21, disrupted the coding region of the gene and an additional six somatic mutations were identified in *MCC* in sporadic colon tumours: four amino acid substitutions and two splice site alterations (Nishisho et al., 1991; Kinzler et al., 1991a). The link between G proteins and colon cancer is strengthened by studies of the anticancer drug sulindac. Sulindac is a cyclooxygenase inhibitor and has been shown to cause the regression of benign tumours in patients with FPC and GS (Waddell et al., 1989; Labayle et al., 1991). G proteins have been shown to regulate phospholipase 2A activity in the generation of arachidonic acid

from phospholipids, a process requiring cyclooxygenase (Jelsema and Axelrod, 1987; Kurachi et al., 1989). *MCC* was therefore considered to be an important gene in colorectal tumorigenesis and a candidate for the gene responsible for FPC.

However, no germline mutations of *MCC* have been found in FPC patients. Furthermore, the discovery of another gene close to *MCC* on 5q21 termed *APC* (see below) which is mutated in the germline of FPC patients has meant that *MCC* is no longer considered a candidate for the FPC gene. In a recent study from our own laboratories of non-selected sporadic colorectal tumours, it was shown that allele losses on 5q21 invariably involved both *MCC* and *APC*, neither gene being lost individually (Curtis *et al* 1994). In addition, in those cases which demonstrated LOH in *MCC*, no mutations of the remaining *MCC* allele were found although these would have been expected if *MCC* were a conventional oncosuppressor gene disabled in both alleles in these tumours (Knudson 1985). Also, few mutations of *MCC* in sporadic colorectal cancers have been identified, whereas mutations of the *APC* gene are common in both FPC and sporadic colorectal tumours. The role of *MCC* in colorectal cancer, therefore, remains uncertain.

## 2.32 The *APC* gene

The second gene to be identified on 5q21 was found by studying the region of 5q21 linked to FPC. Linkage analysis had shown that 5q21 markers were tightly linked to the development of adenomas in many FPC kindreds (Bodmer et al., 1987; Leppert et

al., 1987; Dunlop et al., 1990; Meera-Khan et al., 1989). Two approaches were used to identify genes in this region of 5q21. The first involved the use of cosmid markers to the region to screen YAC libraries. Mapping experiments showed that the coding region of *MCC* was outwith the region of deletion seen in a FPC kindred, suggesting that another gene was responsible for the disease. The *MCC* gene was then used to generate YAC clones to identify genes near *MCC* that were involved in the deletion. Cross hybridisation studies followed by cDNA walking identified a nucleotide transcript and the gene was called *APC* (for adenomatous polyposis coli) (Kinzler et al., 1991b). The second approach involved the characterisation of nested deletions in FPC patients. This study yielded three genes in the deleted region. *MCC* was again shown to be outside the region of deletion. One of the genes termed DP2.5 was shown to be the same as *APC* (Joslyn et al., 1991).

The gene was shown to encode a large 2843 amino acid protein with coiled coil regions in the N terminus and one other coiled coil region in the central portion. The presence of these heptad repeats like those observed in *MCC* suggested the formation of  $\alpha$  helices and the first 55 amino acids of APC have been shown to be sufficient to form stable parallel dimers suggesting that APC may form dimers with itself (Joslyn et al., 1993). Local similarities to myosins, intermediate filament proteins such as desmin and vimentin were found. Database searches also identified homology to the ral2 yeast protein implicated in regulation of *ras* and, like *MCC*, the m3 muscarinic acetylcholine receptor. The genomic structure of *APC* was identified and shown to have an

unusually large fifteenth exon. (Kinzler et al., 1991b; Groden et al., 1991). Reverse transcription PCR (RT-PCR) showed that *APC* was expressed in normal colonic mucosa, a variety of other human tissues, both foetal and adult, and also in cell lines from both sporadic and FPC tumours (Kinzler et al., 1991b). Wild type *APC* has been localised by antibody staining throughout the cytoplasm and colocalised with microtubules. Staining is more intense in the basolateral margins of epithelial cells and increases in the upper portions of the crypt, suggesting an increase in expression of *APC* with maturation of the cell. By contrast mutant *APC* presents as a diffuse cytoplasmic stain (Smith et al., 1993; Smith et al., 1994). Immunoprecipitation experiments revealed that *APC* associates with  $\beta$ -catenin (Rubinfeld et al., 1993; Su et al., 1993a).  $\beta$ -catenin is involved in *adherens* junctions which mediate cell adhesion and may be responsible for the contact inhibition signal.  $\beta$ -catenin also shows homology to plakoglobin and the *Drosophila* armadillo protein which is thought to be part of the signalling pathway for the cell/cell signalling protein Wingless (McCrea et al., 1991). The binding of *APC* to catenins may regulate transmission of the contact inhibition signal. Loss of contact inhibition and the forming of inappropriate cell/cell adhesions are characteristics of neoplastic cells.

Once the gene had been found, its involvement in FPC was established by the identification of mutations in the germline of FPC patients (Nishisho et al., 1991; Groden et al., 1991). Both RNase protection assays and SSCP (single strand conformational polymorphism) analysis identified mutations in the germline of both



FPC and Gardner's Syndrome patients. The *APC* gene was therefore considered to be the gene responsible for FPC. The majority of mutations produced truncating stop codons. Additionally several somatic truncating mutations were identified in sporadic tumours some of which had no loss of 5q21 (Nishisho et al., 1991; Groden et al., 1991). Many studies have since identified *APC* mutations in adenomas and carcinomas of both FPC and sporadic colorectal tumours. One study showed 67% of FPC patients had germline mutations in *APC* with 68% of these clustered in the 5' half of exon 15 (Miyoshi et al., 1992a). The remaining 23% cases without apparent *APC* mutation were provisionally explained as due to lack of sensitivity in detection and mutations occurring in regions of the gene not studied. The majority of mutations discovered in *APC* result in a predicted truncated protein product either by nucleotide substitutions creating stop codons, or small deletions leading to frameshifts. Most mutations occur at CpG or CpA dinucleotides in both germline and somatic mutations. The frequency of point mutations and frameshifts is similar for both germline and somatic mutations. Greater than 60% of somatic mutations of *APC* are clustered in a region of exon 15 (codons 1286-1513) that accounts for less than 10% of the coding region and which has been termed the MCR (for mutation cluster region) (Miyoshi et al., 1992b). Two small deletions account for 15-20% of mutations seen in *APC* in FPC and sporadic tumours (Groden et al., 1993). One of these mutations is in a tandem repeat AAAAG which creates a frameshift and subsequent truncation. The repeat may be a target for deletion by polymerase slippage during replication or unequal sister chromatid exchange (Miyoshi et al., 1992a;

Varesco et al., 1993; Ichii et al 1993). Variations were found in adenoma density, size, distribution, age of onset and extracolonic manifestations in patients with this same *APC* mutation. Additionally carcinomas were shown to occur in the proximal colon as well as the more usual rectosigmoid colon. Therefore this mutation at codon 1309 accounts for intra- and interfamilial phenotypic variation. Additionally identical mutations were observed with variable phenotype in FPC patients (Paul et al., 1993). This suggests the involvement of other factors either genetic or epigenetic that influence the disease phenotype. Interestingly, an identical mutation of a C-T at codon 302 caused Gardner's Syndrome in one patient but no evidence of extracolonic abnormalities was seen in another patient (Paul et al., 1993). Somatic mutations in adenomas from FPC patients were shown to cluster in small regions of within 15bp of codon 1490 and within 24 bp of codon 1429. This clustering may represent regions of differing carcinogen sensitivity within the colon and evidence for this is provided by the study of *APC* mutations in gastric cancers which show a different spectrum and distribution of mutations compared to colon tumours. Alternatively the germline mutation may affect the normal copy of the gene by 'directing' the second hit, or the nuclear location of the *APC* gene may be fixed, thereby 'fixing' the regions exposed to carcinogen (Nakatsura et al., 1992; Ichii et al., 1993).

An attenuated form of FPC termed AAPC (for attenuated adenomatous polyposis coli) is characterised by fewer adenomas than FPC but with a high rate of relatively early carcinoma formation. The AAPC locus has also been mapped to the FPC locus

(Spirio et al., 1993). The *APC* mutations identified were truncations like FPC but were close together and more 5' than those seen in FPC but separated from the most 5' FPC mutations by only 10bp. This may represent a functional boundary between FPC and AAPC.

This less severe phenotype may be explained if mutant *APC* protein complexes with wild type in a dominant negative fashion. In this case the mutations in AAPC may produce a protein incapable of binding to, or inactivating, the wild type copy of the protein. Evidence for this is provided by the finding that truncated *APC* associates with wild type *APC in vivo* (Su et al., 1993b), APC products of less than 80kd are not seen (Smith et al., 1993) and that the first 55 amino acids are sufficient for homodimer formation (Joslyn et al., 1993). But this theory does not explain the large deletions of *APC* seen in FPC. Phenotypic variation due to the location of an *APC* mutation is evident in FPC patients where mutations after exon 9 are often associated with CHRPE (Olschwang et al., 1993).

The number of polyps has also been linked to the location of germline mutations with profuse adenomas common in tumours with mutations within codons 1250-1464 (Nagase et al., 1992). A recent study showed *APC* mutation in 60% of sporadic carcinomas and 63% of adenomas including very small polyps (5mm) therefore suggesting that *APC* mutation is an early event in colorectal tumorigenesis. Some studies suggest that the loss of both copies of the gene is necessary for adenoma

formation (Ichii et al., 1993). Other studies, however, suggest that adenomas may form even if a normal copy of *APC* remains (Miyoshi et al., 1992b; de Benedetti et al., 1994; Miyaki et al., 1990). This apparent conflict remains unsolved.

Germline mutations of the *APC* gene have been found in three cases of Turcot's Syndrome (Mori et al., 1994). Somatic mutations were also seen in the adenomas of these patients but not in the CNS tumours (*APC* mutations have not been detected in sporadic CNS tumours). This suggests that *APC* is important in colorectal tumours but not the associated CNS tumours which may suggest that Turcot's Syndrome is a random association between *APC* mutation and CNS tumours and not an incompletely penetrant variation of FPC as previously reported.

Losses of *APC* are not seen in adenomas from FPC patients but losses arise in the carcinomas of these patients with the same frequency as that seen in sporadic tumours (Vogelstein et al., 1988; Sasaki et al., 1989). The inherited mutation in the adenomas of FPC patients has led to 2 theories concerning the fact that mutation of one copy of the gene is sufficient to allow adenoma formation. The first involves a threshold effect in which the wild type copy of *APC* in adenomas produces enough protein to maintain a non malignant phenotype (Bodmer et al., 1987). The second theory is that the microenvironment of the adenoma in the colonic crypts is important. All cells in FPC patients will contain the mutation, therefore perhaps presenting less of an inhibitory effect on the adenoma development than normal cells in the colon of an individual

with a sporadic adenoma (Paraskeva and Williams 1990). Additionally, *APC* mutations have also been seen in gastric cancers, especially well differentiated adenocarcinomas, and early gastric adenomas (Nakatsura et al., 1992; Tamura et al., 1994).

A murine model of FPC exists in the form of the *min* (for multiple intestinal neoplasia) mouse (Moser et al., 1990). These mice have a nonsense mutation in the murine *APC* gene induced by the chemical carcinogen ENU (ethyl nitrosourea) which cosegregates with the disease phenotype (Su et al., 1992). Heterozygous mice die at around day 120 (dependent on the genetic background) from widespread anaemia, perhaps caused by the presence of multiple adenomas within the intestine. Homozygous *min* mice die *in utero*. There is also evidence for an *APC* modifier locus resulting in a decrease in the number of adenomas and an increased lifespan in the mice (Moser et al., 1992).

In summary, a region of chromosome 5 has been shown to be commonly deleted in many colorectal tumours of both sporadic and inherited origin. Two genes have been identified within the region of loss. One, *APC* has been shown to be the gene for FPC and is also commonly mutated in sporadic tumours as an early event. *APC* has been shown to bind to proteins involved in cell/cell and cell/basement membrane adhesion and signalling. The other gene, *MCC* has been shown to be mutated in only a small number of sporadic tumours and its role in colorectal tumorigenesis is unclear.

## 2.4 Chromosome 12, the *K-Ras* oncogene

The *ras* genes comprise a large gene superfamily including *H-ras*, *K-ras* and *N-ras*, encoding cytoplasmic membrane bound signal transduction proteins with GTPase activity. Single base pair mutations in either codon 12, 13 or 61 activate the gene and when mutated, *ras* can transform immortal cells in culture and also co-operate with other genes to transform diploid primary cultured fibroblasts (reviewed in Bos 1989). Somatic mutations in codon 12 of *K-ras* occur in 9% of sporadic adenomas greater than 1cm in diameter, 22% of adenomas 1-2cm in diameter, 60-75% of adenomas greater than 2cm and 50-65% of carcinomas but in only 7% of all FPC adenomas (Bos et al., 1987; Forrester et al., 1987; Farr et al., 1988; Vogelstein et al., 1988). In tumours that contain both adenomatous tissue and contiguous carcinoma, the same mutation is present in carcinomatous tissue as in the adenoma, therefore, suggesting that *ras* gene mutations precede malignancy. This is analogous to the mutations observed in the *H-ras* gene in skin papillomas and malignant carcinomas chemically induced by dimethylbenzanthracene and tumour promoters (Quintanilla et al., 1986).

**In summary, the *K-ras* gene is commonly mutated as an early event in colorectal tumorigenesis in one of three codons 12, 13 or 61. Different mutations within these codons may relate to prognosis and survival.**

## 2.5 Chromosome 8

### 2.51 The *c-myc* gene

*C-myc* is the proto-oncogene analogue of the avian retrovirus MC29 *v-myc* gene. Elevated levels of the c-myc transcripts are seen in colorectal tumours especially left sided cancers e.g. FPC and some sporadic tumours (Rothberg et al., 1987; Sikora et al., 1987). However, the increase in c-myc has been associated with the increase in ornithine decarboxylase observed in colorectal tumours and may therefore represent an increase in the number of cycling cells rather than over-expression of c-myc itself (Calabretta et al., 1985).

### 2.52 8p

The short arm of chromosome 8 shows losses of 2 regions, 8p23.2-22 and 8p21.3-11.22 in 42% of colorectal cancers and associated with clinicopathological stage (Fujiwara et al., 1993). This may indicate the presence of 2 tumour suppressor genes in this region. However other studies indicate a similar region of loss but with no associated relationship to site or stage (Cunningham et al., 1993; Yaremko et al., 1994). Losses of 8p have also been identified in liver and lung tumours (Emi et al., 1992).

## 2.6 22q, 1p, and 14q

Loss of the long arm of chromosome 22 is recorded in 20-50% of sporadic colorectal tumours (Vogelstein et al., 1989) and have been associated with lymph node metastases in sporadic tumours (Iino et al., 1994).

42% of sporadic colorectal carcinomas show a loss of 1p usually involving the region 1p35 and this frequency of loss is higher in metastases (Leister et al., 1990). Additionally, a series of cell lines derived from an adenoma removed from a FPC patient has genetic changes within this region (Williams et al., 1993). This is intriguing as this region possibly corresponds to the region in the murine genome that contains a modifier of the murine *APC* gene, *Mom-1* for modifier of *Min* (Dietrich et al., 1993).

53% of colorectal tumours have losses in 14q but no losses have been observed in adenomas. There is a significant correlation between 14q loss and site, with tumours of the distal colorectum associated with 14qLOH (Young et al., 1993).

**In summary, loci other than 17p, 18q and 5q are also sites for losses in colorectal cancers. The role of these other loci and genes within them is less well characterised than the previously described genes.**

## 2.7 DNA Repair Enzymes and Genome Instability

Mutations of genes involved in the replication and repair of DNA damage have been suggested to be critical in tumorigenicity (Loeb et al., 1974). Genetic instability has been shown to predispose to cancer in diseases such as ataxia telangiectasia,



xeroderma pigmentosum and Bloom's syndrome. This has led to suggestions that genes involved in DNA repair and replication may be targets for mutation themselves.

### **2.71 DNA polymerase $\beta$**

Mutations in the catalytic domain of the DNA repair gene DNA polymerase  $\beta$  have been identified in a small group of colorectal tumours when compared to normal tissue, placental tissue and blood controls (Wang et al., 1992). This gene is involved in repair of chemically damaged DNA (Wang et al., 1991).

### **HNPCC and instability**

Recently, DNA repair genes have been associated with HNPCC as well as some cancers previously considered to be without an inherited predisposition.

### **2.72 2p**

No linkage has been found between HNPCC and any tumour suppressor genes previously implicated in colorectal cancer. Linkage was shown however to a microsatellite marker on chromosome 2 (Aaltonen et al., 1993; Peltomaki et al., 1993a). Microsatellites are short repeated sequences scattered throughout the genome. They are highly polymorphic between individuals and are therefore highly informative as markers for linkage studies. However, unlike loci containing established suppressor genes such as *APC*, no losses of chromosome 2 were observed in either familial or sporadic tumours. However, microsatellite DNA at the

chromosome 2 marker varied in length from tumour to tumour, often at  $[CA]_n$  or  $[CAG]_n$  sites and this variation was apparent at other sites studied. Another group also reported microsatellite instability in colorectal tumours (Thibodeau et al., 1993). The fact that so many changes had been reported at many different sites made it highly unlikely that they had arisen independently and suggested a widespread infidelity in DNA replication at microsatellite sites. This phenotype was termed RER+ (for replication error). 13% of sporadic tumours were RER+, and these tumours had similar biological properties to familial RER+ cancers. They tended to be right-sided tumours and were all either diploid or near diploid (Aaltonen et al., 1993).  $[CA]_n$  shifts were studied on chromosomes 5q, 15q, 17p and 18q and all showed variation in length in 28% of tumours (Thibodeau et al., 1993). These shifts also showed correlation with tumours of the right side of the colon, were associated with an increased survival rate and inversely correlated with LOH on 5q, 17p and 18q. The mutation rate of CA shifts was shown to be 100 times higher in a small number of RER+ tumour cell lines when compared to RER- cells consistent with the association of a defect in strand specific mismatch repair (Parsons et al., 1993). The latter mutator phenotype has been postulated to be recessive and to represent loss of function of a protein involved in recognising or repairing simple repeat mismatches in DNA (Thibodeau et al., 1993). Traditional cloning methods using polymorphic markers to 2p16 identified a region containing the HNPCC locus (Fishel et al., 1993). Another approach was also used to link mismatch repair and HNPCC. The same region was cloned by virtue of its sequence homology to bacterial and yeast mismatch repair

genes, the *E.coli mutS* and the *S.cerevisiae MSH2* genes respectively (Leach et al., 1993). *MutS* has been shown to encode a protein that recognises mismatches and it has been proposed that replicative DNA polymerases have a high slippage rate on simple repeat sequences, which are repaired by the mismatch repair machinery (Modrich et al., 1991; Kunkel 1993). Defects in this machinery could therefore be responsible for some of the high levels of instability seen in neoplasia. Germline mutations of the gene termed *hMSH2* were detected in HNPCC kindreds and somatic mutations were also found in RER+ tumour cells (Fishel et al., 1993). Additionally, microsatellite instability has been shown in sporadic gastric and endometrial cancers which are characteristics of the HNPCC syndrome, but not lung, breast or testicular tumours which have no association with HNPCC (Peltomaki et al., 1993b).

## 2.73 3p

Recently a second gene involved in mismatch repair has been identified and shown to be located on the short arm of chromosome 3 (Papadopoulos et al., 1994; Lindblom et al., 1993). This gene has shown homology to another mismatch repair protein, the homologue of the *E.coli MutL* gene termed *hMLH1*. *MutL* is thought to encode a protein that stabilises the MutS protein and an endonuclease (the MutH protein product). Both studies identified mutations of *hMLH1* in HNPCC patients. The replacement of chromosome 3 into colon cell lines with homozygous mutations in *hMLH1* reduced mismatch repair and microsatellite instability (Koi et al., 1994).

Two additional homologues of MutL have since been identified, hPMS1 and 2, located on chromosomes 2 and 7 respectively (Nicolaidis et al., 1994). Inactivation of any of the three MutL-related genes can result in HNPCC. Microsatellite instability has also been observed in other tumour types such as oesophagus and chronic myelogenous leukaemia (Meltzer et al., 1994; Wada et al., 1994; Horii et al., 1994). )

**In summary, it has been suggested that mutations in several of the genes involved in DNA mismatch repair and replication may cause the genomic instability and high mutation rate observed in HNPCC and some sporadic colorectal tumours.**

## **2.8 DNA Hypomethylation**

DNA methylation is a covalent modification of the mammalian genome that occurs almost exclusively at CpG dinucleotides and approximately 70% of cytosine bases are methylated in normal differentiating cells (Doerfler et al., 1983). Changes in methylation occur during normal differentiation and in primary tumours (Feinberg et al., 1988). Hypomethylation may predispose genes to expression and may inhibit chromosome condensation leading to mispairing and non-disjunction (Groudine et al., 1981; Harrison et al., 1983). Hypomethylation is a characteristic of human colonic tumours and often precedes malignancy. DNA from colorectal adenomas and carcinomas is selectively hypomethylated to a similar degree when compared to normal tissue (Goelz et al., 1985; Feinberg et al., 1988).

In summary, there are a number of recognised common genetic events implicated in the genesis of colorectal tumours involving both oncogenes and tumour suppressor genes. Some of these such as mutation of the *APC* gene and *ras* oncogene are generally considered early events involved in adenoma formation and progression. Others, including mutation and deletion of the *p53* and *DCC* genes are more closely associated with adenoma to carcinoma transition and carcinoma progression. Recent identification of errors in the genes controlling mismatch repair pathways has suggested a novel mechanism for some colorectal tumour development. Whilst many of the genes involved in colorectal carcinogenesis are common to both sporadic and inherited forms of the disease the way the various gene products interact and function in disease progression is still unclear.

## **2.9 The Clinical Relevance of Genetic Abnormalities observed in Colorectal Cancer.**

Many studies have attempted to relate specific genetic abnormalities to clinicopathological grade of tumour. This has lead to a certain level of confusion with some studies apparently contradicting others. However, there are general comparisons to be made between genetic lesions and tumour pathology although the meaning of these correlations is not always clear.

Specific lesions have been related to specific situations. For example, losses of 17p have been correlated with vascular invasion in one study (Iino et al., 1994). This finding seems at odds with the previously reported role of *p53* in the transition from adenoma to carcinoma (Nigro et al., 1989). *p53* mutations have also been shown to occur in a small subset of adenomas regardless of size and whether from sporadic or familial patients (Baker et al., 1990b). Additionally, studies of *p53* mutation in both the oesophagus and brain indicate that this is an "early" event in these tumour types (Bennett et al., 1991; Sidransky et al., 1992). This suggests that whilst in the majority of colon tumours loss of *p53* function by mutation and LOH occurs during or around the conversion to malignancy, in other tumour types and in a subset of colon tumours *p53* mutation may occur either prior to or post-malignant conversion. This may reflect different pathways in neoplastic progression and suggests that loss of *p53* is a preferred "early" event in colorectal tumorigenesis, but is not essential for malignant conversion. Mutations of *p53* are often observed in the context of a destabilised genome, with DNA amplifications and aneuploidy common (Yin et al., 1992; Livingstone et al., 1992). It is unclear, however, whether it is the *p53* mutation itself that causes the instability or whether the mutation allows the survival of cells with gross chromosomal changes that would normally be eliminated by *p53*-mediated control and which become progressively more unstable. An early loss of *p53* function could render an adenoma genetically unstable and by removing a *p53*-induced block to malignant progression allow the accumulation of other critical genetic lesions involved in tumour progression. A study of colon cancer metastases showed that the

presence of a mutant p53 protein, regardless of whether a wild type protein was also present, correlated with lymphatic spread, whereas loss of the wild type allele (LOH) correlated to haematological spread and distant metastases (Goh et al., 1994). However, this does not prove that the p53 loss with or without concomitant retention of the wild type allele, was causative of the metastatic behaviour of the tumour. In general it has been noticed that colorectal tumours with *p53* mutation are usually more aggressive and relate to a shorter survival (Yamaguchi et al., 1992). This is, perhaps, consistent with the idea of widespread genomic instability, although not all studies agree with this idea. (Starzynska et al., 1992; Hamelin et al., 1994). Additional evidence for the increased instability potentially afforded by p53 mutation can be seen from studies that show *p53* mutation more commonly in tumours with 17p LOH. Further evidence comes from studies of familial breast cancer (FBC). FBC has a higher association with *p53* mutation than sporadic breast tumours. FBC tumours also show changes in number of dinucleotide repeats on chromosome 17 suggesting that DNA replication and repair defects induced by *p53* mutation may create instability at least in this tumour type (Glebov et al., 1994).

The spectrum of *p53* mutations seen in different tumour types may reflect the differing cellular environments and responses to carcinogens. Thus the timing of *p53* mutation may simply be a reflection of the genetic background of the cell with respect to carcinogen sensitivity.

A recent study suggests that tumours with losses of 18q may be associated with a poorer survival rate than those with no 18q loss (Jen et al., 1994). The losses that generally occur late in tumour development i.e. during malignant progression, such as 18q LOH linked to liver metastases and 22q to lymph node metastases may reflect the ability of invading metastatic cells to implant at different sites (Itoh et al., 1993; Iino et al., 1994). Another study suggests that changes in *DCC* are related to the degree of lymph node metastasis and degree of differentiation in oesophageal squamous cell carcinoma (Miyake et al., 1994). However, this could simply represent tissue specific differences.

There is also evidence for inactivation of *APC* leading to the accumulation of mutations or abnormalities in other genes and at other sites such as *ras*, *p53* and 8p. In one study, the frequency of *APC* mutation observed in adenomas from FPC patients was the same regardless of size or histopathology but the frequency of *K-ras* mutation increased in adenomas with increasing dysplasia and size. Additionally, 17p and 8p losses are generally rare events in adenomas but common in carcinomas (Ichii et al., 1993). In contrast, another study suggested that the frequency of *APC* mutations was higher in tubulovillous and villous adenomas than tubular adenomas, indicating a link with histological type (de Benedetti et al., 1994). A tendency was observed for the frequency of *APC* mutations observed in colon tumours to increase with increasing loss of differentiation. However, in gastric tumours *APC* mutation occurs in well differentiated cancers (Nakasuru et al., 1992; Miyaki et al., 1990). This



suggests that *APC* abnormalities may occur later in tumour progression in some sporadic colorectal cancers.

The question of APC involvement in all cases of colorectal tumours is unclear. For example the literature is divided on whether both alleles of *APC* need to be inactivated in the formation of adenomas or whether disruption of one allele is sufficient. Some studies show some adenomas with one allele of *APC* inactivated and other adenomas with both alleles disrupted (de Benedetti et al., 1994). The question also remains as to whether mutations of *APC* function as dominant negative mutations or whether mutations result in reduced amounts of *APC* protein perhaps below a threshold level required for normal function. If mutations in *APC* function in a dominant negative manner, adenomas with a mutation in only one allele may nonetheless have no or reduced APC function. Additional loci are very probably involved in the modification of APC in most colorectal tumours. This is evident in cases of FPC in which identical APC mutations result in differing phenotypes in different individuals.

The relevance of specific *K-ras* mutations is also unclear. A study by Shibata suggested that certain *ras* mutations present in adenomas may be more likely to cause progression to a carcinoma (Shibata et al., 1993). Other studies which have looked at the relevance of *ras* gene mutations have shown a high association between codon 12 mutations and colorectal cancer and suggested that codon 12-val and codon 13-aspartate mutations are found in tumours limited to the bowel wall, whereas other mutations,

particularly codon 12-asp may be associated with metastasis (Finkelstein et al., 1993). In contrast, in another study, G-T and G-C transversions e.g. 12-ala and 12-val have been associated with Dukes C colorectal tumours with metastases (Moerkerk et al., 1994). This recent study also identified multiple point mutations in codon 12 in some tumours associated with increasing stage of the tumour. Another study however has found no correlation between *ras* mutation and metastasis (Roehlitz et al., 1993). Additionally it has been reported that codon 12 and 13 mutations except 12-asp are more common in patients with recurrent disease (Benhatter et al., 1993) and may be directly involved in tumour progression in a dominant manner (Forrester et al., 1987). *Ras* mutations are less common in flat adenomas (typically only 16%) and no significant difference in mutation spectrum was seen between flat or polypoid adenomas and carcinomas (Minamoto et al., 1994). However, the correlation between *ras* mutations and clinicopathological variables remains unclear.

In general, sporadic tumours and those associated with FPC tend to be located in the distal and sigmoid colon and rectum (left sided), whereas tumours associated with HNPCC and flat adenoma syndrome tend to be located in the caecum and ascending colon (right sided). The basis of this site bias is unknown but may be related to differing concentrations of faeces and carcinogens as well as responses to gut carcinogens at different sites along the colon. Left and right sided cancers may have important clinical distinctions as they can differ in both mucus composition and associated blood group antigens (Yonezawa et al., 1982; Denk et al., 1974). Losses of

5q 14q and 18q are more common in left sided tumours (Iino et al., 1994; Young et al., 1993).

If it is accepted that a number of carcinomas arise from pre-existing adenomas then a general model for tumour progression may be proposed.

1. *APC* mutation results in a.) the formation of mutant/wild type heterodimers and b.) Probable adenoma formation.
2. LOH wt*APC* and *K-ras* mutation result in the progression to a moderately or severely dysplastic adenoma.
3. LOH 17p leads to the formation of intramucosal carcinomas.
4. LOH 18/22q results in invasive carcinomas

This model suggests that LOH increases with increasing dysplasia (Miyaki et al., 1990) and is consistent with the model proposed by Fearon and Vogelstein (Fearon and Vogelstein, 1990). However whilst this model may account for perhaps the majority of colorectal cancers, evidence presented earlier shows that situations arise in which this model is not applicable.

A novel mechanism for colorectal tumorigenesis is evident in colorectal cancers from HNPCC families, in which a relatively normal karyotype of diploid or near-diploid is commonly observed (Kouri et al., 1990). Sporadic RER+ tumours have less chromosome losses and *p53* mutation than sporadic RER- tumours, in contrast to the familial breast tumour data presented earlier (Thibodeau et al., 1993; Aaltonen et al.,



1993; Glebov et al., 1994). Cells in adenomas are usually diploid or near-diploid whereas those of most colorectal carcinomas contain aneuploid subclones. Aneuploidy increases with size and histopathological type of adenoma but not dysplasia (Quirke et al., 1987; Hiddemann et al., 1986; Jarvis et al., 1987). A decreased incidence of aneuploidy is observed in cases of adenocarcinoma in association with adenoma compared with colorectal cancer in general. *p53* mutation has been shown to precede and possibly initiate the divergence of aneuploid subclones in colorectal tumours (Carder et al., 1993; Carder et al., 1994). Cells with abnormal *p53* are at a higher risk of replication of the entire genome prior to completion of cell division, leading to tetraploidy via endoreduplication (Carder et al., 1993). This suggests at least 2 ways to generate the genetic heterogeneity that is observed in colorectal neoplasia. The first involving gross chromosomal changes and losses leading to a highly aneuploid phenotype, involves *p53* in the generation and maintenance of the instability. This suggests that only cells that have lost *p53* remain viable due to the otherwise catastrophic effects of such widespread genetic damage. This model is possibly that seen in FPC and some sporadic tumours. The second pathway involves small multiple changes throughout the genome created by errors in mismatch repair, whilst retaining a diploid or near diploid karyotype-like tumour observed in HNPCC families and some sporadic cases.

**In summary, many of the genetic abnormalities associated with colorectal tumorigenesis have been correlated with clinicopathological variables. There is**

still however much confusion in the literature regarding the role some of these lesions play in carcinogenesis. Some of the questions raised above will be addressed in the following chapters, including the role of MCC in colorectal neoplasia, and the effect *APC* mutation as an early event plays in colorectal cancer progression. Additionally, the relationship between mutation of the *p53* gene and stabilisation of the protein will be studied.

## Chapter 3

### The relationship between *p53* mutation and protein stabilisation in colorectal carcinomas.

#### 3.1 Introduction

In normal cells, p53 protein is expressed at a low level and has a short half-life (6 minutes in the spleen, Rogel et al. 1985). A change in molecular configuration may lead to stabilisation of the protein so that its half life is greatly extended, thereby allowing detection by immunocytochemistry (ICC). A number of human and murine antibodies to p53 exist, some of which identify both wild type and mutant configurations, whilst at least one antibody is specific for the mutant conformation alone. (Jenkins et al. 1985; Gannon et al., 1990). These antibodies have been applied to both Western analysis and Immunoprecipitation studies of p53. Their use in distinguishing between mutant and wild type protein on denatured, fixed sections in ICC is, however, not so certain. Many studies have shown the presence of stabilised mutant p53 protein in different tumour types by immunocytochemistry (Cattoretti et al., 1988; Bártek et al., 1990; Iggo et al., 1990; Van den Berg et al., 1989; Rodrigues et al., 1990; Marks et al., 1991; Gusterson et al., 1991; Purdie et al., 1991). Many of the tumour-associated missense mutations in *p53* occur in cases with stabilised protein and it has often been assumed that stabilised p53 visualised by ICC is equivalent to mutated *p53*. Recently however, it has become clear that the association between

stabilisation of the protein and mutation of the gene is far from absolute (Wynford-Thomas, 1992). Both false negatives and positives have been reported. Gross deletions or nonsense mutations may alter translation of the protein, although truncating mutations are rare except in some sarcomas. Alternatively point mutations may fail to stabilise the protein to a level detectable by ICC and may or may not still retain some normal function, both of which result in a false negative (Børresen et al., 1991; Rodrigues et al., 1990). False positives have been identified in clonal cell lines on which the argument that insensitivity of the mutation detection technique fails to locate a mutation are inappropriate (Rodrigues et al., 1990; Lehman et al., 1991). The relationship between mutation and stabilisation may reflect both the type of mutation and the nature of the cell in which it occurred. A study of *p53* in 33 lung cancer cell lines with no normal *p53* revealed a correlation between missense mutations in exons 5-8 of the gene and strong overexpression of the protein, whilst a low expression of protein correlated with deletion, splicing or nonsense mutations as well as missense mutations outside exons 5-8 (Bodner et al., 1992). Conversely, not all *p53* mutations result in a stabilised protein product. For example, Li-Fraumeni patients carry germline *p53* mutations yet mutant protein is detectable in only a proportion of cases (Frebourg et al., 1992). Not all stabilised protein is mutated. Wild type *p53* protein levels rise after DNA damage as the natural response to agents such as UV irradiation and the protein assumes a stable configuration detectable by ICC (Hall et al., 1993). Some cancer family patients express stabilised wild type *p53* protein in a substantial proportion of their cells (Barnes et al., 1992). An enzyme-linked immunoassay has

recently shown that *p53* stabilisation occurs in some colorectal adenomas in the absence of mutation but not in surrounding normal mucosa (Tominaga et al., 1993). This *p53* protein is not in the mutant conformation and may be located within the cytoplasm. This may suggest the presence of a subset of colorectal tumours in which loss of *p53* function, perhaps due to retention within the cytoplasm, occurs as an early event. A study of *p53* staining in breast cancers revealed cytoplasmic staining of wild-type *p53* protein in 37% of cases, suggesting that nuclear exclusion may be a further mechanism in the inactivation of *p53* tumour suppressor activity in some tumours (Moll et al., 1992). Finally there is evidence to suggest that wild type *p53* protein can be stabilised through the activation of other genes already implicated in cancer, such as *c-myc* and *ras*. Under these circumstances, wild type *p53* function may be abrogated without mutation of the gene (Lu et al., 1992). Recently it has been suggested that the tumour cell environment may impose a stable configuration on wild type protein, perhaps causing the loss of its normal growth regulatory responses (Vojtešek and Lane, 1993).

This study has attempted to determine the frequency with which mutation and stabilisation occur in colorectal tumours. There are particular reasons for studying *p53* in colorectal cancer in this way.

Firstly it has been shown that the presence of stabilised *p53* correlates with a particular class of cancers in which there are multiple divergent, near-tetraploid



tumour cell clones and the suggestion has been made that such abnormal *p53* may facilitate endoreduplicative events leading to aneuploidy (Carder et al., 1993).

Secondly, there are interesting comparisons to be made between primary cancers of the colorectum and at other sites. Existing evidence suggests that *p53* can be involved in carcinogenesis in different ways. Thus, both colorectal and breast cancer show frequent stabilisation, but point mutation frequency in breast cancer is relatively low, commonly 20-38% (Bártek et al., 1991; Dunn et al., 1993; Jacquemier et al., 1994) whilst in existing series of colorectal cases, the incidence of *p53* point mutations is greater than 50% (Rodrigues et al., 1990; Nigro et al., 1989). Overexpression of *p53* protein without mutation in high grade non-Hodgkin's lymphomas has also been demonstrated (Villuendas et al., 1993).

Thirdly, *p53* ICC in colorectal tumours, as in other cancers, shows a variety of staining patterns. In some, staining is intense and affects the majority of nuclei (often associated with mutation) (Rodrigues et al., 1990; Baas et al., 1994), in others it is widespread but involves fewer nuclei (Lu et al., 1992), and in some there is only focal staining that may affect a very low proportion of nuclei (often not associated with mutation and may represent a focal response to local DNA damage) (Vojtešek et al., 1993; Baas et al., 1994; Fritsche et al., 1993). Alternatively, the presence of sporadic staining nuclei may represent the emergence of new clones within the tumour and this has been seen within adenomas (Kikuchi-Yanoshita et al., 1992). Widespread staining involving few nuclei is also seen with wild type *p53* (Bártek et al., 1990; Midgley et al., 1992; Casey et al., 1991; Gannon et al., 1990). High *p53* expression also

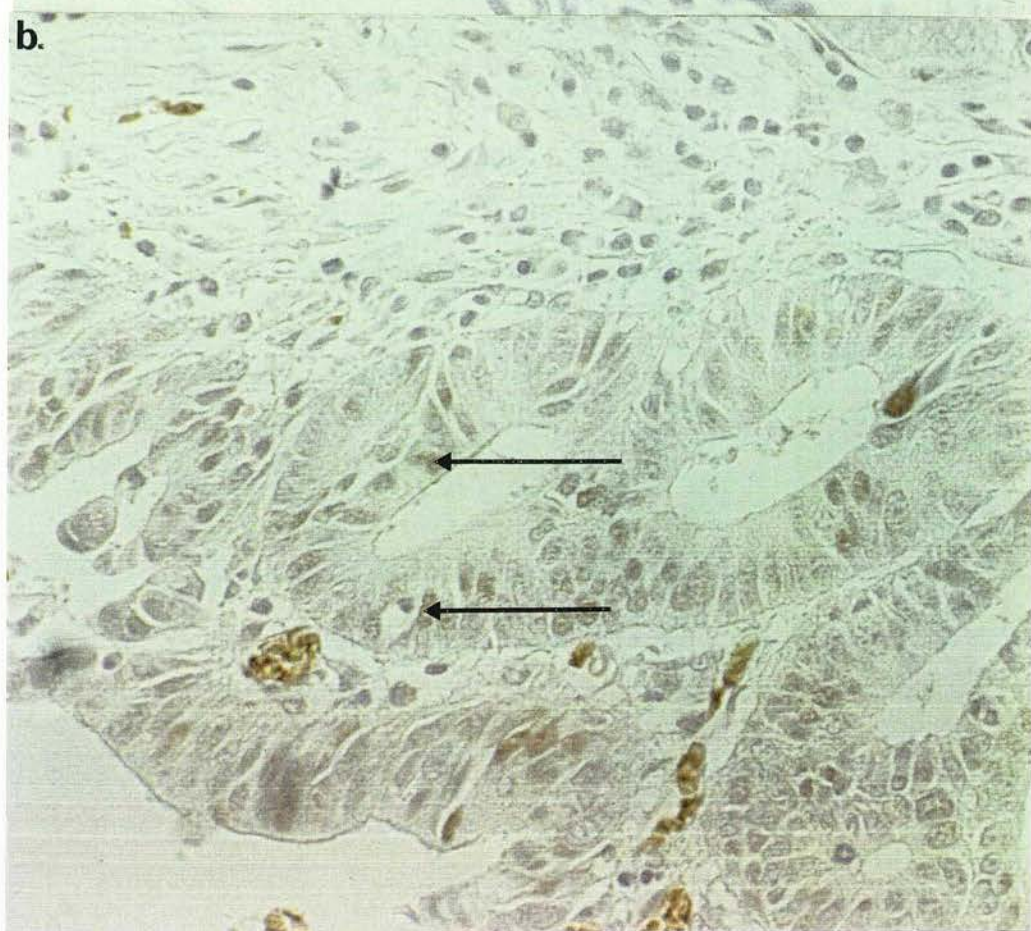
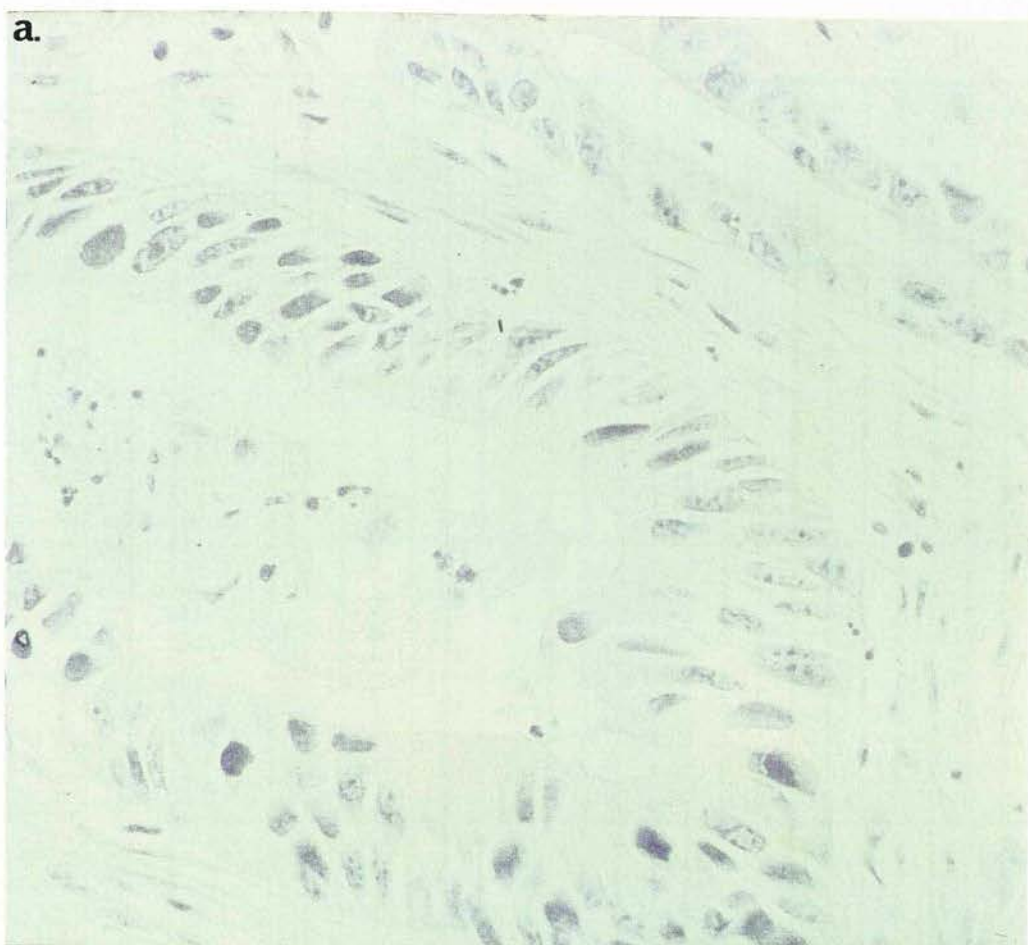
correlates with cells showing abnormal cell cycle control and mitotic failure, suggesting that carcinoma cells may well have high levels of genome instability (Lu et al., 1992; Kastan et al., 1991). Different patterns of staining probably represent a different biological basis for the expression of *p53* protein.

In an attempt to resolve some of these questions, 47 colorectal carcinomas, originally classified as positive or negative dependent on staining with the *p53* monoclonal antibody PAb1801 (Purdie et al., 1991; Carder et al., 1993), were examined. Mutations were defined in ICC positive cases by sequencing through exons 5-8, the region in which over 90% of all *p53* mutations have been shown to occur in previous studies (Levine et al., 1991). A method for screening for mutations using PCR-SSCP and an *MspI* restriction enzyme digest was then validated with ICC positive cases and applied to ICC negative cases. Finally, the 47 cases were re-examined with a second antibody, DO7, under conditions which were more sensitive in detecting stabilised *p53*. Mutations outside exons 5-8 were sought. In the few cases with consistent, strongly positive nuclear staining with both antibodies but no evidence of mutation within exons 5-8, sequence data were determined for the remainder of the gene. The combined data support a strong relationship between stability and mutation, but highlight specific situations where stability and mutation are not concordant.

**Figure 2. PAb1801/immunoperoxidase detection of p53 in colorectal carcinoma specimens.**

- a.) Immunocytochemically negative colorectal carcinoma.
- b.) Immunocytochemically positive colorectal carcinoma. Nuclear staining of p53 protein is indicate by arrows and was present in the majority of tumour cells within this section.

(Approximately x200)



**Table 1. A COMPARISON BETWEEN LOCATION OF MUTATION IN 23 TUMOURS AND IMMUNOCYTOCHEMISTRY WITH PAB1801.**

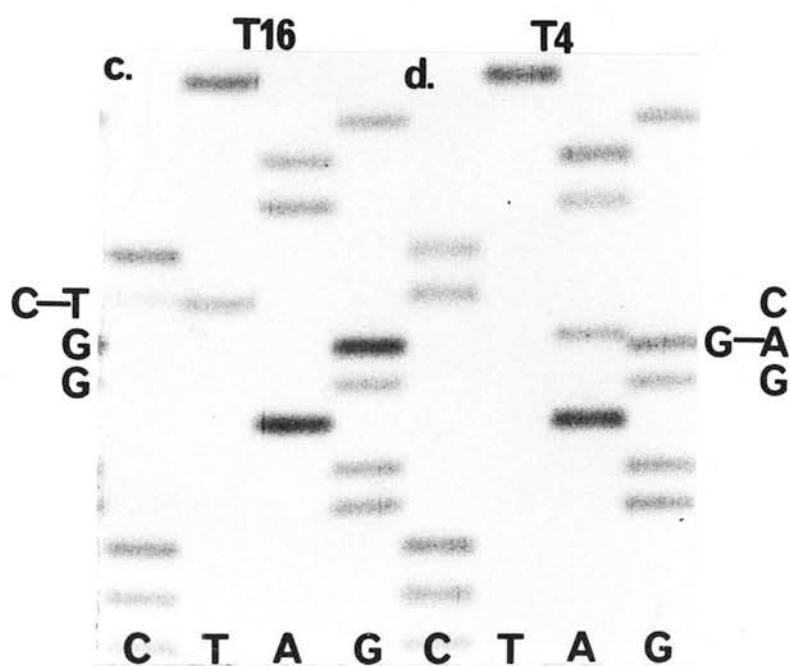
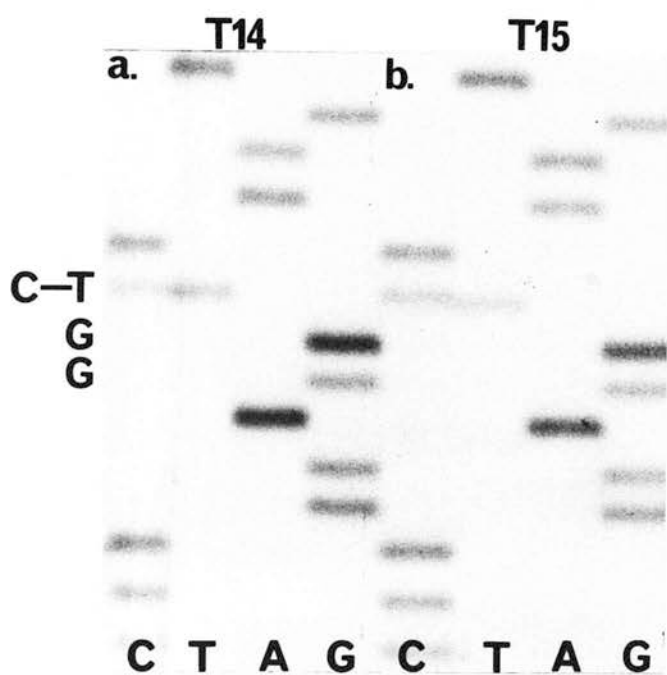
Case		IMMUNOCYTOCHEMISTRY POSITIVE		IMMUNOCYTOCHEMISTRY NEGATIVE		AMINO ACID CHANGE
		SSCP POSITIVE	SSCP NEGATIVE	SSCP POSITIVE	SSCP NEGATIVE	
T1	EXON 5	codon 152 CCG-CTG				Pro-Leu
T2		codon 175 CGC-CAC				Arg-His
T3		codon 175 CGC-CAC				Arg-His
*T4		codon 175 CGC-CAC				Arg-His
T5				codon 175 CGC-CTC		Arg-Leu
T6		codon 179 CAT-TAT				His-Tyr
T7		codon 180 GAG-TAG				Glu-stop
T8			exon/intron 5 Ggt-Gat			
T9	EXON 6			codon 202 CGT-CAT		Arg-His
T10				codon 203 GTG-ATG		Val-Met
T11				codon 213 CGA-TGA		Arg-stop
T12	EXON 7		codon 227 TCT-TCG			Ser-Ser
T13		codon 245 GGC-AGC				Gly-Ser
T14		codon 248 CGG-TGG				Arg-Trp
T15		codon 248 CGG-TGG				Arg-Trp
T16		codon 248 CGG-TGG				Arg-Trp
T17		codon 248 CGG-TGG				Arg-Trp
T18		codon 248 CGG-TGG				Arg-Trp
*T4			codon 248 CGG-CAG			Arg-Gln
T19			codon 248 CGG-CAG			Arg-Gln
T20			codon 248 CGG-CAG			Arg-Gln
T21			codon 248 CGG-CAG			Arg-Gln
T22	EXON 8	codon 281 GAC-AAC				Asp-Asn

All individual tumours in which a mutation was detected by sequencing except \* in which the 2 mutations were detected in the same tumour. In addition T23, a tumour negative for immunocytochemical staining and for SSCP in exons 5-8 contained a TTC-TCC (Phe-Ser) mutation at codon 113

**Figure 3. Sequence analysis of exon 7 of the p53 tumour suppressor gene in colorectal tumours.**

- a.) Sequence analysis of exon 7 of p53 in tumour T14 showing a CGG-TGG (Arginine to Tryptophan) mutation at codon 248.
- b.) Sequence analysis of exon 7 of p53 in tumour T15 showing a CGG-TGG (Arginine to Tryptophan) mutation at codon 248.
- c.) Sequence analysis of exon 7 of p53 in tumour T16 showing a CGG-TGG (Arginine to Tryptophan) mutation at codon 248.
- d.) Sequence analysis of exon 7 of p53 in tumour T4 showing a CGG-CAG (Arginine to Glutamine) mutation at codon 248.

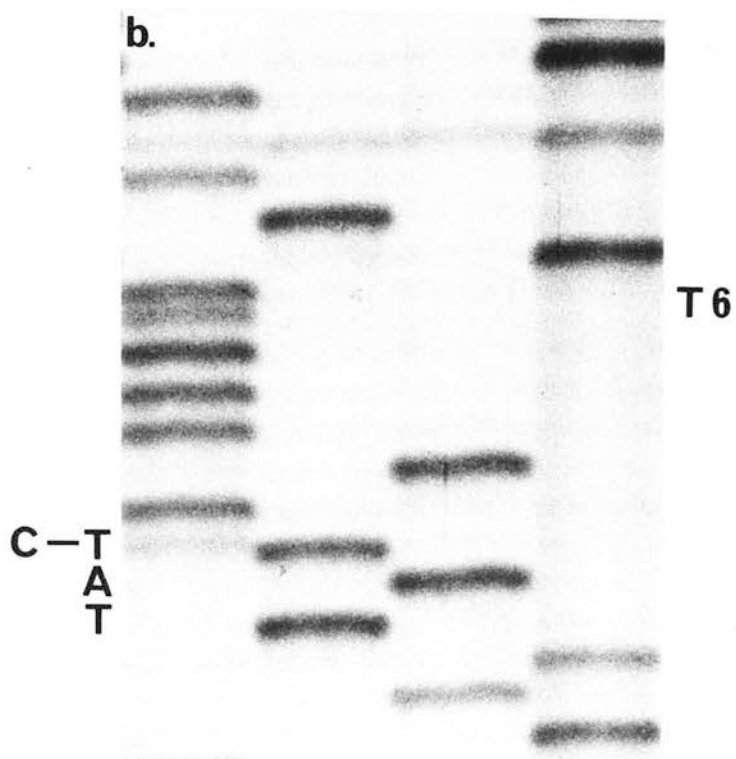
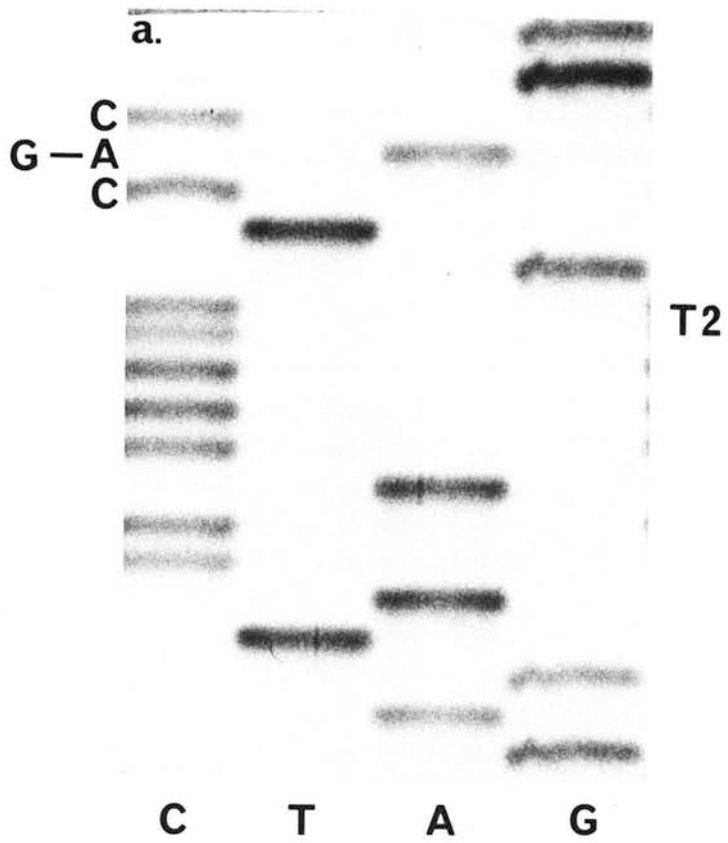
The presence of bands indicating the wildtype sequence reflects contaminating stromal (normal) tissue in the tumour sample.



**Figure 4. Sequence analysis of exon 5 of the p53 tumour suppressor gene in colorectal tumours.**

- a.) Sequence analysis of exon of p53 in tumour T2 showing a CGC-CAC (Arginine to Histidine) mutation at codon 175.
- b.) Sequence analysis of exon of p53 in tumour T6 showing a CAT-TAT (Histidine to Tyrosine) mutation at codon 179.





## **3.2 Results**

### **3.21 Mutation analysis of PAb1801 positive tumours**

Paraffin sections of 47 colorectal carcinomas fixed in PLPD and previously identified as either immunocytochemistry positive or negative (ICC+ or ICC-), were restained with the antibody PAb1801 as previously described (Purdie et al., 1991)(Figure 2). In this analysis a tumour was classified as ICC positive if *any* nuclear staining was observed within the section and so included all 3 staining patterns previously described. Positive staining was observed in 27 of the tumours. Sequencing of exons 5-8 of the *p53* gene in these cases revealed 19 mutations in 18 (67%) of the tumours (Table 1 and Figures 3 and 4). Interestingly, one case, (T4), contained 2 different mutations, CGC→CAC in codon 175 and CGG→CAG in codon 248. T4 had no 17p allele loss detectable using a number of polymorphic markers (data not shown) and therefore the two mutations were presumed to be in different alleles. Two mutations in the same gene are rare but have been previously reported in the literature. The majority of mutations (12/19) clustered within 2 recognised hotspots at codons 175 and 248 (Hollstein et al., 1991).

In one case, (T8), a mutation was found at an exon/intron boundary (G→A of the first base of intron 5), whilst in another, (T12), a conservative base change at codon 227 was detected. Of 19 mutations, 17 were either C→T or G→A transitions, 13 occurring at CpG dinucleotides. The remaining 2 cases were a G→T and a T→G

transversion. The nature of these mutations is therefore in agreement with the published literature in which the majority of *p53* mutations in colorectal cancer are base pair transitions occurring at CpG dinucleotides within recognised hotspots (Hollstein et al., 1991). Of the 27 tumours with stabilised *p53*, 9 (33%) did not contain a mutation within exons 5-8. In order to determine whether this was due to the presence of mutations outwith exons 5-8, the entire coding region was sequenced in the 3 tumours showing the strongest staining of the majority of nuclei with PAb1801. These were considered to be the most likely to contain mutation of the gene. A mutation in exon 4 was detected in one of the tumours resulting in a phenylalanine to serine amino acid change at codon 113 (data not shown). Mutations in exon 4 and 9 of the *p53* gene have previously been reported in the literature and therefore exons 4 and 9 were sequenced in the remaining 6 ICC+ cases but no further mutations were found. Therefore 8/27 (30%) cases with positive PAb1801 immunocytochemistry appear to have no mutation at least within the regions studied which comprise the most commonly mutated regions.

### **3.22 PCR-SSCP/MspI screening for mutations in PAb1801 positive tumours**

Rather than search for mutations in all the PAb1801 negative cases by sequencing, a more rapid but accurate screening method based upon SSCP of exons 5-8 was developed. This method derived from that of Orita et al., 1988 but did not use radioactive isotope. The basis of SSCP analysis is that, upon electrophoresis of single

stranded DNA through a non-denaturing gel, the DNA will adopt a secondary conformation dependent upon its base sequence. Mutation of even a single base can alter the secondary structure and in a proportion of cases allow their detection by comparison of abnormally migrating DNA bands and corresponding 'normal' samples. In this experiment PCR products were not radiolabelled and the DNA was visualised in the gel by a silver staining technique. To validate the method it was first applied to the PAb1801 positive tumours where the sequence within exons 5-8 was known. 13/19 (68%) of the sequence-detected mutations revealed an abnormal band migration pattern under standard SSCP condition, whilst 6 mutations were not detectable by this method (Figure 5). One of these was the exon/intron boundary mutation in tumour T8, which would have been masked by the PCR primers used to amplify exon 5 for SSCP analysis. The conservative base change at codon 227 was also undetectable as an SSCP. The remaining 4 cases were all codon 248 CGG→CAG mutations. SSCP analysis was repeated under different conditions of 40°C in the absence of glycerol, but no further mutations were detected. In order to extend this screening method to include reliable detection of the common codon 248 mutations, a PCR fragment containing exons 7-9 was digested with the restriction enzyme MspI. Disruption of the CCGG MspI recognition site at codon 248 by mutation resulted in the loss of 135bp and 168bp bands and addition of a 303bp band (Figure 6). The combination of SSCP and MspI digestion detected 89% of mutations known to exist in exons 5-8 on the basis of sequencing. Moreover, all SSCP abnormalities were confirmed as mutations; that is, there were no false positives.

**Figure 5. Single Strand Conformational Polymorphism analysis of exons 5, 7 and 8 of the p53 gene amplified by the polymerase chain reaction in immunocytochemically positive colorectal tumours.**

In each pair of lanes, normal DNA (on left) is compared with tumour DNA from the same patient (on right). Mutations detectable as bands of altered mobilities are marked by arrows.

Lanes 1 and 2 Tumour T22: Exon 8.

Lanes 3 and 4 Tumour T19: Exon 7.

Lanes 5 and 6 Tumour T17: Exon 7.

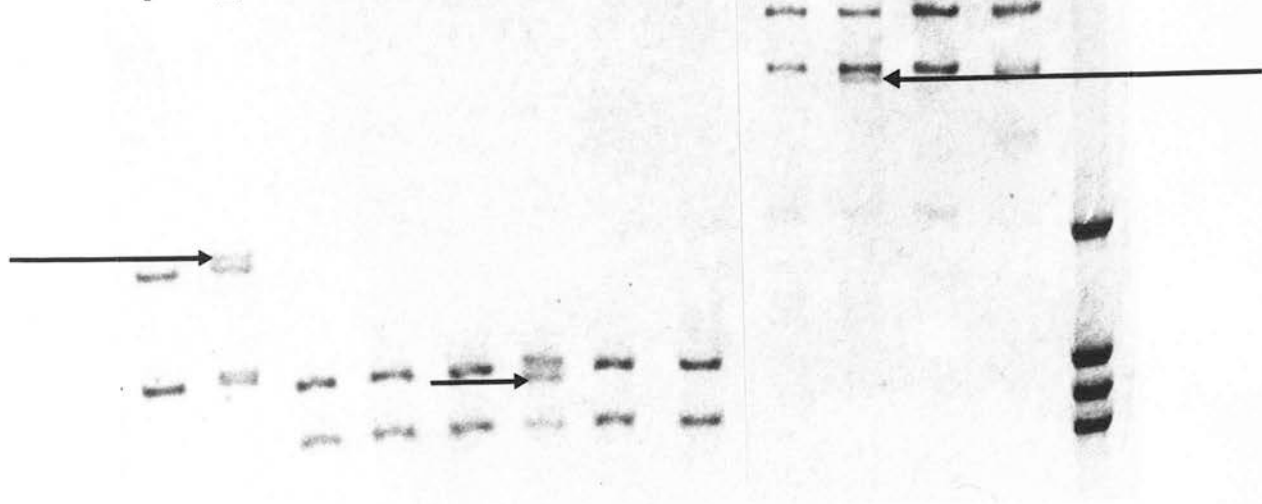
Lanes 7 and 8 Tumour T4: Exon 7.

Lanes 9 and 10 Tumour T4: Exon 5.

Lanes 11 and 12 Tumour T3: Exon 5.

Lane 13 DNA marker V (Boehringer Mannheim)

1 2 3 4 5 6 7 8 9 10 11 12 13



**Figure 6. Agarose gel of MspI digested exons 7-9 PCR amplified DNA identifying exon 7 codon 248 mutations of p53.**

0.8% agarose gel stained with ethidium bromide

Lanes 1 and 2 Normal DNA sample and corresponding tumour T4

Lanes 3 and 4 Normal DNA sample and corresponding tumour T19.

Lanes 5 and 6 Normal DNA sample and corresponding tumour T15.

Lane 7 and 8 Normal DNA sample and corresponding tumour T13.

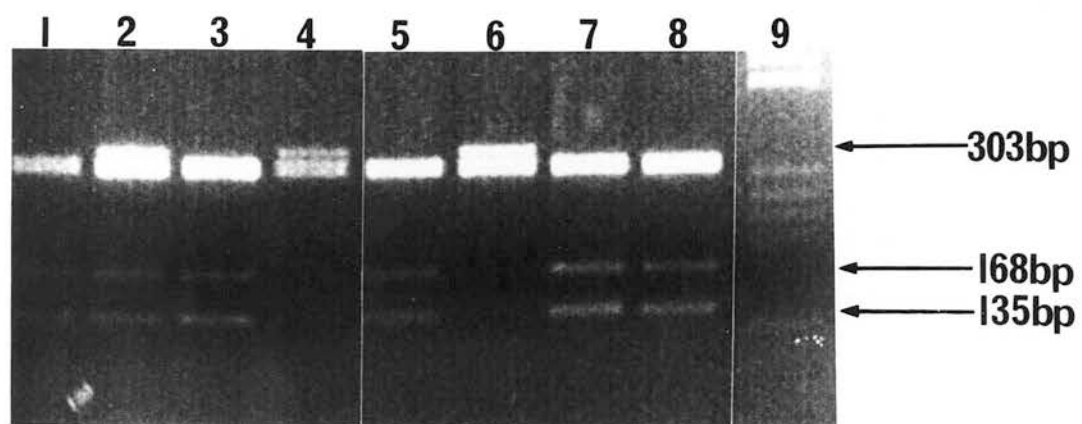
Lane 9 DNA Marker VI (Boehringer Mannheim)

Tumours T4 and T19 contained G-A mutations at codon 248 which were undetectable as bands of altered mobility on SSCP analysis. In contrast tumour T15 contained a C-T mutation at codon 248 that was detectable by SSCP analysis.

Mutation at codon 248 disrupts a MspI restriction digest site observed as an extra band of 303 base pairs on digestion of a PCR fragment containing exons 7-9 of the p53 gene with MspI. Contaminating normal DNA in tumour sample T4 is identified by the retention of 135 and 168 base pair bands which are identifiable in all the normal DNA samples presented. Tumour T13 contained a mutation at codon 245 and hence did not reveal the loss of an MspI site at codon 248. The constant bands at approximately 280 and 290bp are due to the presence of two internal MspI restriction enzyme sites unaffected by the presence or absence of a mutation at codon 248.

N-normal DNA sample.

T-tumour DNA sample.





### 3.23 Screening for mutations in PAb1801 negative tumours

Of the 47 tumours studied, 20 were classified as ICC negative. In these, no PAb1801 staining was seen within the section (Figure 2). Nonetheless, when these tumours were studied by the SSCP/MspI screening method, 4 tumours (20%) showed an abnormal SSCP band pattern when compared to the corresponding normal sample (Figure 7). These abnormalities were confirmed by sequencing. One of the mutations was at codon 175 within exon 5, whilst the remaining 3 were all in exon 6 at codons 202, 203 and 213. In contrast, no exon 6 mutations were found amongst the PAb1801 positive cases (Table 1).

### 3.24 DO-7 and CM-1 Immunocytochemistry

It was possible that failure to identify stabilised product in the presence of mutated *p53* merely reflected the sensitivity of the method of staining. To address this, the sections were restained under optimised conditions of microwave pre-treatment with the human *p53* specific murine monoclonal antibody DO-7 (Cattoretti et al., 1993). Both PAb1801 and DO-7 showed similar patterns of staining with the 3 classes of staining pattern discussed above being readily discernible (data not shown). Although microwave pre-treatment and DO-7 staining produced fewer negative cases than PAb1801 (9 versus 20), the 4 PAb1801 negative cases with mutation were also all clearly negative with DO-7. The epitopes recognised by PAb1801 and DO-7 are both within the N-terminal domain of *p53* (Banks et al., 1986; Vojtešek et al., 1992) and it

**Figure 7. Single Strand Conformational Polymorphism analysis of exons 5, 6, 7 and 8 of the p53 gene amplified by the polymerase chain reaction in immunocytochemically negative colorectal tumours.**

Mutations detectable as bands of altered mobilities are marked by arrows.

Lanes 1 and 2 Exon 5  
Lanes 3 and 4 Exon 5  
Lanes 5 and 6 Exon 8  
Lanes 7 and 8 Exon 7  
Lanes 9 and 10 Tumour T9: Exon 6  
Lanes 11 and 12 Exon 8  
Lanes 13 and 14 Tumour T5: Exon 5  
Lanes 15 and 16 Tumour T10: Exon 6  
Lanes 17 and 18 Exon 7  
Lane 19 DNA marker VI (Boehringer Mannheim)

Lanes 1, 2, 3, 4, 5, 6, 7, 8, 11, 12, 17 and 18. Normal (N) and tumour (T) DNA from immunocytochemically negative tumours with no p53 mutation detectable.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19



was therefore possible that both antibodies might have failed to detect a genuinely stabilised protein through some epitope masking phenomenon. To exclude this, the 3 cases with exon 6 mutations that were negative with both antibodies were stained with the polyclonal antibody CM-1. No positive staining was seen, although CM-1 convincingly detected stabilised protein in 3 further known DO-7 positive cases included in the same run (data not shown). Finally, no cases with single scattered positively staining nuclei with either PAb1801 or DO-7 contained mutations, but mutations were found in 2 cases with only 30-50% moderately strongly positive nuclei on staining with DO-7. As mentioned earlier, 2 tumours in which the majority of nuclei stained strongly with both PAb1801 and DO-7 failed to reveal mutations in the entire coding region of the gene.

### **3.3 Discussion**

The data clearly show that positive ICC staining with either DO-7 or PAb1801 does not correlate exactly with *p53* mutation. It is difficult to give absolute figures for this disparity as the proportion of cells staining differs with the method and antibody used and the percentage of positive cases is also dependent upon the extent to which tumours with sparsely or focally distributed positive nuclei are included. Variations are evident in several studies of both cervical and colorectal cancers in which method of fixation, type of antibody and section pretreatment affected the staining patterns observed (Lambkin et al., 1994; Baas et al., 1994). The methods used for ICC will affect the threshold of detection and it has been suggested that the number of cells staining is probably more important than the intensity of stain (Hall and Lane, 1994).

This study shows, however, that mutations can be found in approximately 20% of colorectal carcinomas that are ICC negative, and that mutation in exons 5-8 cannot be found in as many as 33% of ICC positive colorectal cancers. The conservative base change in T12 is unlikely to be responsible for the stabilisation of the protein, which must therefore be attributable either to a second mutational event outside the region studied or to a non-mutational mechanism. The group of ICC positive cancers without apparent *p53* mutation in exons 5-8 contain all the cases in which stabilised *p53* protein was evident only in rare widely-dispersed nuclei. However there were also 3 cases that showed the more familiar staining pattern, with the majority of nuclei strongly positive. One of these was shown to contain an exon 4 mutation at codon 113. Occasional mutations in both exons 4 and 9 have been reported in the literature

and although this was the only mutation recorded outwith exons 5-8 in this study, these observations suggest that previous studies restricted to exons 5-8 may have slightly under-represented the true incidence of *p53* mutations in colorectal tumours. Amongst the mutated cases that were ICC negative, we noted a predominance of mutations in exon 6. It is probable that these tumours contained only mutant protein as data derived by colleagues showed that 2 of the 3 tumours had lost alleles within *p53* whilst the remaining case was non-informative with a variety of polymorphic probes. Previous studies of ICC and mutation in colorectal and ovarian cancers also suggested that exon 6 mutations often failed to result in a stabilised protein (greater than 64% of total cases studies) (Kikuchi-Yanoshita et al., 1992; Cunningham et al., 1992; Teneriello et al., 1993). Taken together these results suggest that exon 6 includes a region of the gene in which mutations frequently fail stabilise *p53*, whilst still conferring a selective growth advantage.

The presence of mutations in occasional ICC negative tumours and the absence of mutations in some strongly ICC positive tumours suggests that alternative mechanisms of *p53* abnormality (stabilisation, mutation or both) occur relatively frequently in colorectal cancers, a tumour type in which identity between stabilisation and mutation has often been assumed. An absence of direct correlation between stabilisation and mutation has been reported in other tumour types including breast carcinoma and non-Hodgkin's lymphoma (Dunn et al., 1993; Villuendas et al., 1993) and high levels of *p53* protein have been associated with poor prognosis in breast

cancer (Thor et al., 1992). Studies on two breast cancer cell lines transfected with a murine mutant *p53* suggested that the mutant murine protein was only stable in a cellular environment where the endogenous gene was stable suggesting that the cellular environment creates the stability and not the mutation *per se* (Vojtesek and Lane, 1993). It has been shown that *p53* protein accumulates in cells with DNA damage (possibly including mutation of *p53* itself), levels of the protein transiently rise, then fall again possibly after repair of the DNA damage. Regulation of mutant *p53* is however altered and so levels of protein may remain high and are therefore 'permanently' detectable by ICC. In this case the cellular environment of the cell (i.e. the DNA damage) stimulates *p53* overexpression and mutation of *p53* itself may render the protein incapable of accurately responding to DNA damage. If this is the case, the *p53* overexpression seen in tumours may represent a response to the increasing number of genetic errors seen in the increasingly unstable DNA and not to the mutation of the gene itself. Alternatively binding to cellular oncoproteins like MDM-2 may stabilise *p53* protein without mutation of the gene, or as in the case of cervical tumours with HPV 16 or 18 infection, *p53* may be degraded by the action of the E6 protein.

As larger case numbers accrue in which the distinction is made between mutational and non-mutational mechanisms of stabilisation including the role of cellular oncoproteins, it should be possible to determine whether this distinction is accompanied by differences in tumour behaviour.

In summary, a high correlation between stabilisation of p53 protein and mutation of the gene has been established in colorectal tumours but specific cases exist in which this concordance is not absolute. These include both cases of ICC- tumours with mutations predominately in exon 6 and ICC+ tumours in which the stabilised product did not seem to be associated with a point mutation of the gene. Positive ICC may therefore generally be accepted as indicative of point mutation within the gene although the lack of complete concordance should be noted.



## Chapter 4

### Is the *MCC* gene involved in Colorectal Carcinogenesis?

#### 4.1 Introduction

As discussed earlier (Chapter 2), several tumour suppressor genes have been implicated in colorectal cancer. A region on chromosome 5q21 is commonly deleted in sporadic colorectal carcinomas and several genes have been mapped to this region including both *APC* and *MCC*. In contrast to *APC*, no mutations of *MCC* have been found in the germline of patients with FPC nor have they been reported in any other inherited colorectal cancer syndrome. Even in apparently large series of sporadic carcinomas, the incidence of somatic mutations of *MCC* is small (Kinzler et al., 1991b; Nishisho et al., 1991). Although the data cast doubt over the general significance of *MCC* mutations in colorectal cancer it remains possible that *MCC* mutations may be important in special groups of colorectal tumours. Of particular interest are cancers that arise in younger patients and those that cluster in family groups. Therefore, the entire coding region of the *MCC* gene was screened for mutations in a small subset of clinically important colorectal tumours from non-polyposis patients who presented with the disease at a young age (under 48 years). By contrast, FPC patients usually present with colon tumours in the second or third decades of life whilst sporadic tumours usually occur in patients over 60 years. Three of the selected patients had a family history of colorectal disease, with one conforming

to the criteria for hereditary non-polyposis colorectal cancer (HNPCC) (Table 2). Tumours had been grown for at least 4 passages as subcutaneous xenografts in SCID mice. This provided a renewable source of tissue, which was free from human stromal elements that could have 'masked' any mutations that may have been present. Mutational analysis was performed by screening for single strand conformational polymorphisms within individual exons (PCR-SSCP) on matching blood and xenograft material as previously described for p53 mutations (Cripps et al., 1994a and Chapter 3).

Table 2. CLINICO-PATHOLOGICAL AND MOLECULAR GENETIC DATA

Patient number	Sex and age (years)	Family History of colorectal disease	Location of tumour	Dukes Stage	5q21 status <sup>a</sup>	MCC exon 10 fragment	MCC exon 14 fragment	MCC exon 15 fragment	p53 ICC	17p status <sup>b</sup>	p53 mutation	Ki-ras (codon 12) <sup>c</sup>
1	male 48	-	Ascending colon	C	loss	A1/A1 <sup>f</sup>	B1/B1	C1/C1	+	loss	cd 248	mutated
2	male 40	-	sigmoid colon	C	loss	A2/A2	B2/B2	C2/C2	-	non-informative	cd175	wild-type
3	male 45	brother	sigmoid colon	C	retained	A1/A2	B1/B1	C1/C1	+	retained	cd248	wild-type
4	male 39	maternal aunt	sigmoid colon	A	loss	A1/A1	B1/B1	C1/C1	-	non-informative	nd <sup>g</sup>	wild-type
5	male 36	father, 2 brothers, grandmother and aunt	unknown <sup>d</sup>	D	retained <sup>e</sup>	A1/A1	B1/B1	C1/C1	-	retained	nd	nd

a.) Previously identified by a variety of polymorphic probes flanking *APC* and *MCC* (Ashton-Rickardt *et al* 1989).  
b.) Established from LOH at YNZ.22 (Matlashewski *et al* 1987) or polymorphic loci within p53 (P. Chumakov pers.comm.)  
c.) Established by sequencing.  
d.) Peritoneal carcinomatosis at operation.  
e.) Retained at the one site informative within the *APC* gene (M. Dunlop pers. comm.)  
f.) A1/B1/C1 refers to the more frequent allele in the population, whilst A2/B2/C2 refers to the less frequent polymorphic allele.  
g.) nd -: not done

## **4.2 Results**

### **4.21 PCR-SSCP of *MCC* in positive controls**

The PCR-SSCP technique used here had previously been employed to screen for mutations of the *p53* gene in a large series of colorectal cancers and detected about 85% of mutations shown to be present by sequence analysis (Cripps et al., 1994a and Chapter 3).

In order to assess the sensitivity of this technique in identifying point mutations in the *MCC* gene, however, DNA from all five of the sporadic colorectal cancers previously reported to have *MCC* point mutations (Nishisho et al., 1991) were screened by PCR-SSCP in the relevant exon. In every case, mutated DNA was detectable through the presence of bands of altered mobility when compared to relevant normal samples. However, in one tumour, two mutations occurred in the same exon and PCR fragment, thus rendering it impossible to determine whether one or both mutations were detectable (Figure 8 and Table 3).

### **4.22 PCR-SSCP of *MCC* in five young patients**

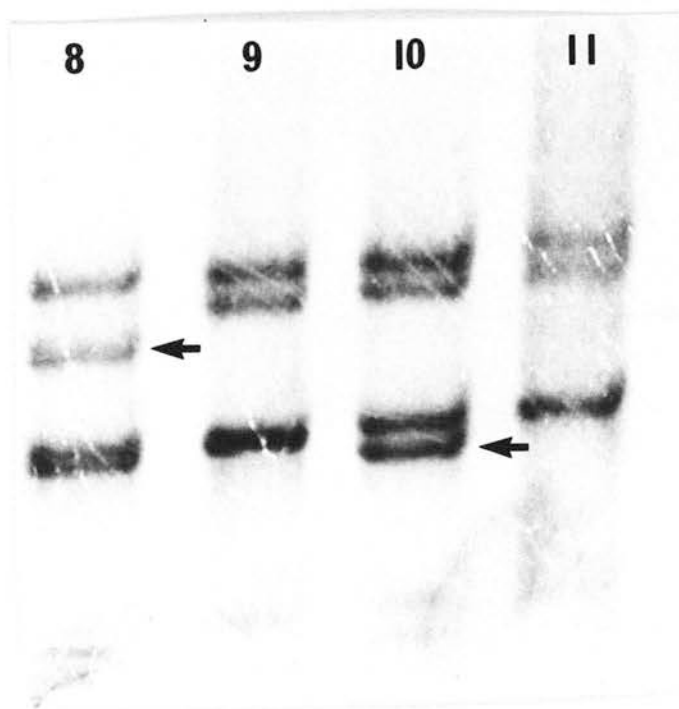
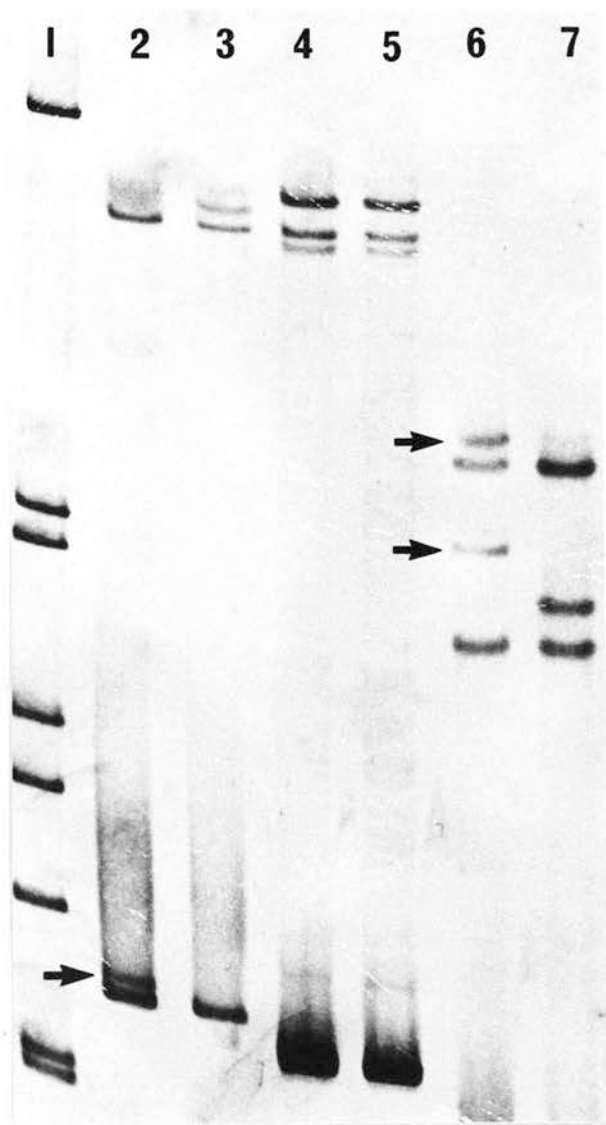
All 17 exons of *MCC* in matching tumour (after maintenance as xenografts in SCID mice) and blood DNA samples were analysed by PCR-SSCP from the five selected young, non-polyposis patients. No mutations were detected in any of the exons in any of the five cases.

**Figure 8. Single Strand Conformational Polymorphism analysis of MCC positive control samples.**

MCC mutations (Nishisho et al., 1991) detectable as bands of altered mobilities compared to appropriate normal samples.

Lane 1	DNA Marker VI
Lanes 2 and 3	T35 and N35: Exon 12
Lanes 4 and 5	T91 and N91: Exon 17
Lanes 6 and 7	T91 and N91: Exon 15
Lanes 8 and 9	T(Sp)83 and N91: Exon 12 (No normal material for T9Sp)83 available).
Lanes 10 and 11	T35 and N35: Exon 12

The apparent band differences between samples T35 and N35 in the 2 gels (lanes 2/3 and 10/11) is believed to be due to variations in running conditions between gels. Differences in band patterns between gels has previously been noticed, however, in both gels the difference assumed to be due to a point mutation between the normal N35 and tumour T35 sample is clear. Reproducibility of SSCP band patterns appears to depend on the gel running conditions (temperature, glycerol, polyacrylamide and TBE concentrations etc.) as well as the PCR fragment studied and may be problematic if the routine detection of specific mutations is required. However, in this study the SSCP technique was used to search for differences between normal and tumour samples (therefore indicating a mutation) and such differences were readily detectable on all gels studied.



**Table 3. POSITIVE CONTROL MUTATIONS IDENTIFIABLE AS SSCP ABNORMALITIES**

MUTATION	TUMOUR	SSCP ABNORMALITY
Exon 12 G-A 1517 Arg-Gln	T35	+
Exon 12 G-C 1793 Gly-Arg	Sp83	+
Exon 13 C-T 1677 Pro-Leu	F8, F17	+
Exon 13 C-T 1753 silent	N35, T35, Sp83	+
Exon 15 C-T 2093 Ala-Val T-C 2124 silent	T91	+

However, three polymorphisms in the PCR fragments containing exons 10, 14 and 15 were found (Figures 9, 10 and 11). The exon 10 fragment polymorphism has been observed by others and contains a 14bp deletion within intron 9 (K. Kinzler, pers. comm.) This polymorphism reportedly present in up to 50% of alleles is evident in two of the five cases, one heterozygous for the polymorphism (termed A1/A2) and the other homozygous (A2/A2). Sequencing of the exon 15 polymorphism confirmed a GCC-GCT silent mutation at codon 708 as previously described (D'Amico et al., 1992)(Figure 12). One patient was homozygous (C2/C2) for this polymorphism (Table 2). The exon 14 PCR fragment polymorphism was found to be a two base pair change within intron 13, immediately prior to exon 14 (Figure 13) and the patient with the homozygous polymorphism in exon 15 was also homozygous (B2/B2) for the intron 13 polymorphism (Table 2). The exon 15 and intron 13 polymorphisms in the five cases, therefore, appeared to show linkage. In order to study this suggested linkage further, SSCP of the PCR fragments containing exons 14 and 15 was carried out on an additional 19 constitutional DNA samples from young, non-polyposis, colorectal cancer patients and 18 normal control DNA samples.

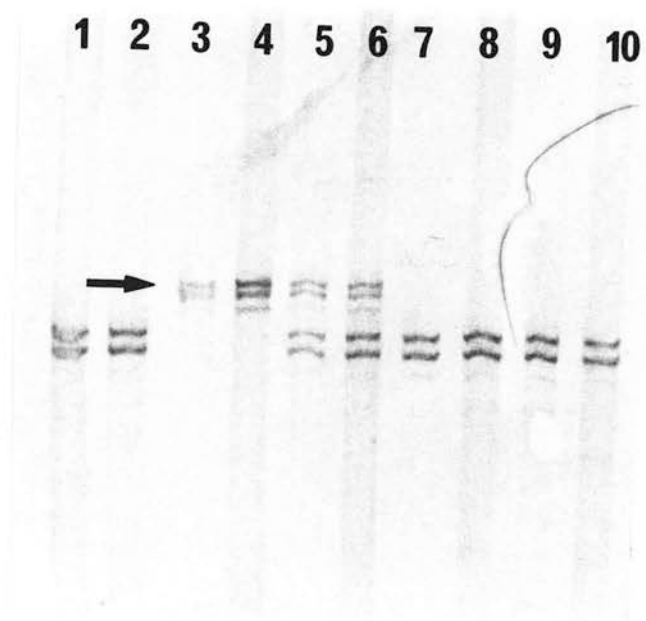
The linkage suggested by the five original cases was confirmed in this larger sample (data not shown). Samples exhibiting heterozygosity or homozygosity for the polymorphism in exon 15 exhibited similar allele status in intron 13. The frequency of



**Figure 9. Single Strand Conformational Polymorphism analysis of exon 10 of MCC.**

Lane 1	Normal (N) DNA sample	}from patient 1
Lane 2	Xenograft (X) DNA sample	
Lane 3	Normal (N) DNA sample	}from patient 2
Lane 4	Xenograft (X) DNA sample	
Lane 5	Normal (N) DNA sample	}from patient 3
Lane 6	Xenograft (X) DNA sample	
Lane 7	Normal (N) DNA sample	}from patient 4
Lane 8	Xenograft (X) DNA sample	
Lane 9	Normal (N) DNA sample	}from patient 5
Lane 10	Xenograft (X) DNA sample	

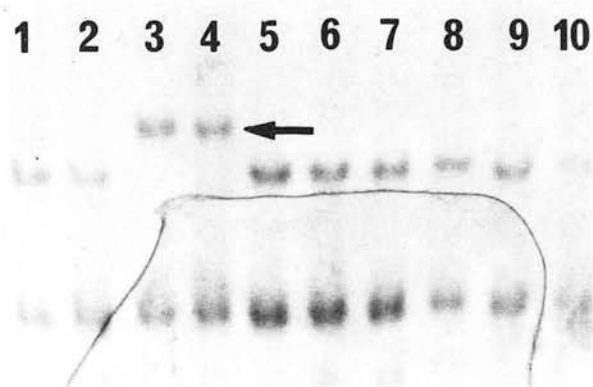
Normal and xenograft samples from patients 2 and 3 contain an exon 10 deletion polymorphism identified as bands of altered mobility compared to patients 1, 4 and 5. Patient 2 is homozygous and patient 3 is heterozygous for the polymorphism.



**Figure 10. Single Strand Conformational Polymorphism analysis of exon 14 of MCC.**

Lane 1	Normal (N) DNA sample	}from patient 1
Lane 2	Xenograft (X) DNA sample	
Lane 3	Normal (N) DNA sample	}from patient 2
Lane 4	Xenograft (X) DNA sample	
Lane 5	Normal (N) DNA sample	}from patient 3
Lane 6	Xenograft (X) DNA sample	
Lane 7	Normal (N) DNA sample	}from patient 4
Lane 8	Xenograft (X) DNA sample	
Lane 9	Normal (N) DNA sample	}from patient 5
Lane 10	Xenograft (X) DNA sample	

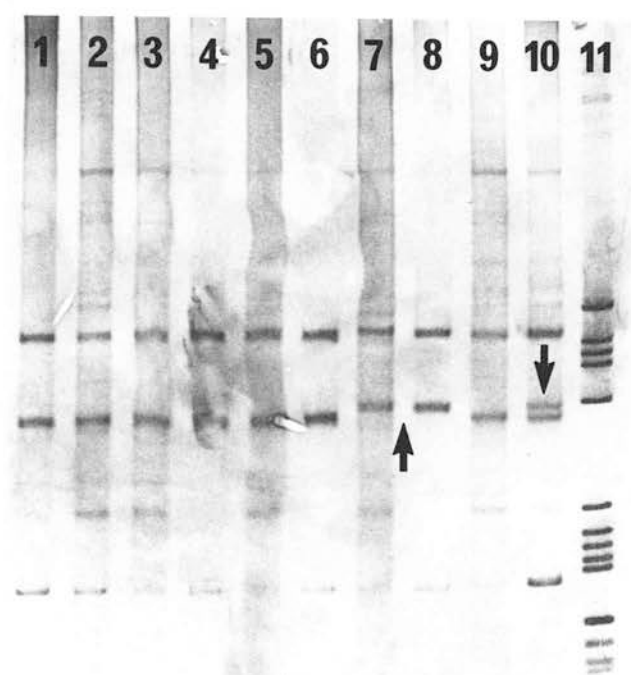
Patient 2 contains a homozygous polymorphism in exon 14 of MCC.



**Figure 11. Single Strand Conformational Polymorphism analysis of exon 15 of MCC.**

Lane 1	Xenograft (X) DNA sample	}from patient 5
Lane 2	Normal (N) DNA sample	
Lane 3	Xenograft (X) DNA sample	}from patient 4
Lane 4	Normal (N) DNA sample	
Lane 5	Xenograft (X) DNA sample	}from patient 3
Lane 6	Normal (N) DNA sample	
Lane 7	Xenograft (X) DNA sample	}from patient 2
Lane 8	Normal (N) DNA sample	
Lane 9	Xenograft (X) DNA sample	}from patient 1
Lane 10	Normal (N) DNA sample	
Lane 11	DNA Marker V (Boehringer Mannheim)	

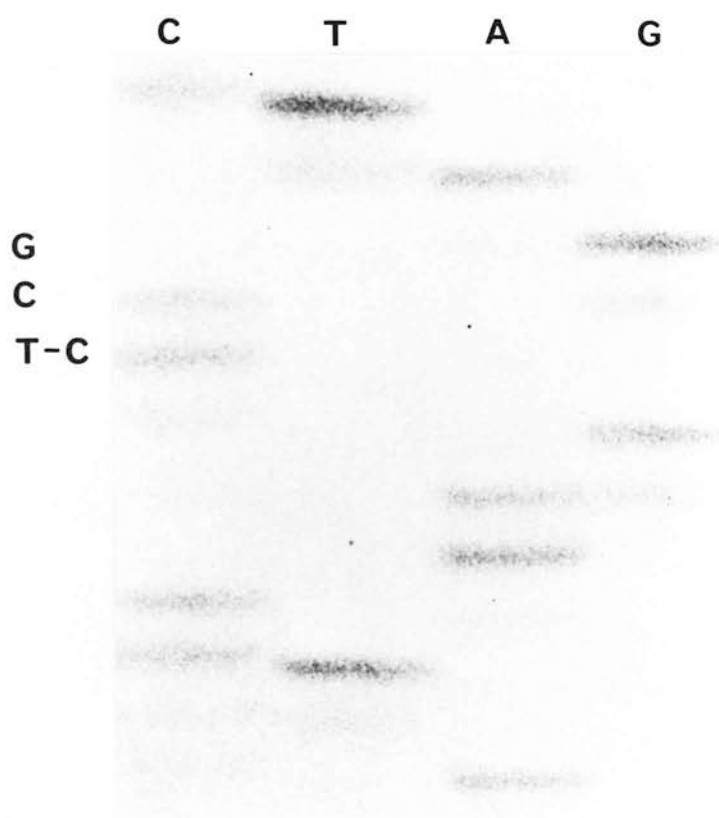
Patient 1 contains a heterozygous and patient 2 contains a homozygous polymorphism in exon 15 of MCC.



**Figure 12. Sequence analysis of exon 15 of the MCC tumour suppressor gene**

Sequence analysis of exon 15 of MCC in the xenograft tumour sample from patient 2 showing a GCT-GCC silent polymorphism at codon 729.

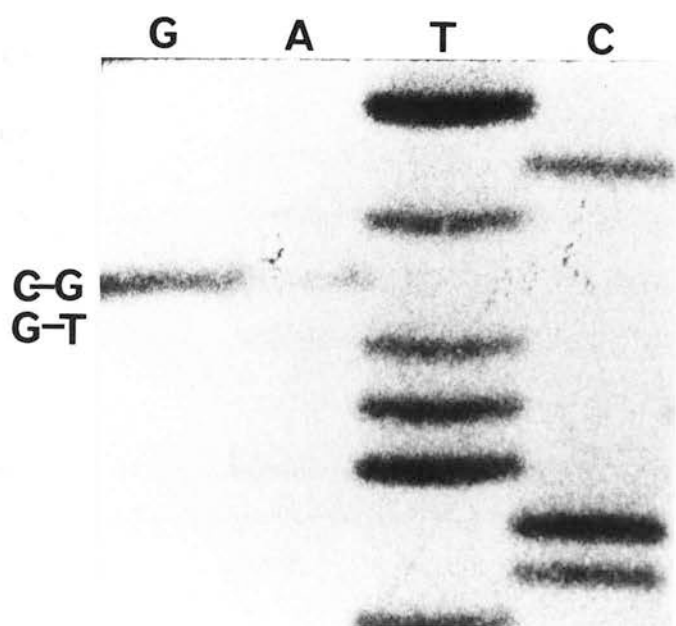
For unknown reasons but assumed to be due in some way to secondary structure, bands in two lanes per position were seen throughout the gel. The darker band has been assumed to be the correct sequence for this tumour and corresponded to the published sequence of the gene except at the position indicated above





**Figure 13. Sequence analysis of intron 13 of the MCC tumour suppressor gene**

Sequence analysis of intron 13 of MCC in the xenograft sample from patient 2 showing a 2 base pair substitution GC-TG at the 10th and 11th bases before the start of exon 14



polymorphisms within the young cancer patient series showed no significant difference to the normal population.

### **4.3 Discussion**

The lack of mutation in the *MCC* gene in this study may be explained in several ways.

A first, trivial, explanation could be that the technique may not have been sensitive enough to detect subtle point mutations within the gene. Although SSCP does not have a 100% detection rate, this explanation is unlikely for several reasons. All the samples with known point mutations were detectable as bands of altered mobilities on SSCP analysis under standard conditions. A previous study of *p53* mutations by the method of PCR-SSCP detected the majority of mutations in *p53* in a large series of colorectal tumours (Cripps et al 1994a and Chapter 3). Additionally, SSCP analysis did detect polymorphisms, including both single and double base pair changes, one of which in intron 13 was previously unreported. This suggests that the technique was sensitive enough to have detected any mutation of *MCC* that might have been present in our series (including splice acceptor/donor alterations).

A second reason for failure to detect mutations in tumour samples may be due to the masking effect of contaminating normal tissue. The use of xenograft material in this study however, eliminated contaminating human stromal elements. The risk of cross contamination with murine stromal tissue was considered negligible due to the use of intron based PCR primers.

Finally, the small sample size may not have been representative of the *MCC* gene in this subset of colon cancers from young patients. This criticism is difficult to dismiss completely, but the following argument is proposed. All other established genetic lesions in colorectal cancer occur with a frequency of around 50% or greater within the appropriate populations e.g. abnormalities of *APC*, *p53*, and *K-ras* in sporadic cancers, and more recently the RER phenotype in HNPCC (Powell et al., 1992; Baker et al., 1989; 1990, Bos et al., 1987; Aaltonen et al., 1993). Were the true incidence of *MCC* mutations similar (i.e. 50%), the chance that 5 independent cases without *MCC* mutation would be drawn at random from such a population would be  $(0.5)^5$  or 0.03, substantially less than the 0.05 level normally accepted as a limit of statistical significance. The data from this small but comprehensively studied series of colorectal carcinoma patients, therefore, do not suggest a major role for *MCC* mutation in colorectal carcinogenesis (Cripps et al., 1994b).

An earlier study which addressed sporadic colorectal cancer in the older age groups also showed that the residual copy of *MCC* was not mutated in a series of 21 cases selected on the basis of allele loss in *MCC* and analysed for mutations by the same PCR-SSCP technique (Curtis et al., 1994). Additionally, evidence against a role for *MCC* in colorectal carcinogenesis comes from studies of patients with FPC in which no mutations of the *MCC* gene have been detected in patients with FPC. No linkage between *MCC* and disease was found in another hereditary form of colorectal cancer, hereditary non-polyposis colorectal cancers (Grodén et al., 1991; Joslyn et al., 1991;

Kinzler et al., 1991b; Nishisho et al., 1991; Peltomäki et al., 1992). More recently, the genes responsible for HNPCC have been shown to be involved in mismatch repair and create microsatellite instability (Aaltonen et al., 1993; Fishel et al., 1993; Leach et al., 1993; Bronner et al., 1994; Papadopoulos et al., 1994).

The above findings, taken together with this study of a small but nevertheless important clinical group of colorectal tumours strongly suggest that *MCC* is not an independently functioning tumour suppressor gene mutated in colorectal cancers at least at the level studied. However its possible role as a modifier gene in this, as well as in other tumour types remains to be established.

**In summary, the results from this and other studies from the laboratory indicate that the *MCC* gene is unlikely to play an important role in colorectal cancer arising in either young non-polyposis patients or the majority of sporadic tumours.**

## **Chapter 5**

### **Targeting of the *APC* gene in a colon carcinoma cell line**

#### **5.1 Introduction**

##### **5.11 The role of APC in normal and neoplastic cells**

There is much evidence to suggest that APC function is essential for normal growth and development. Firstly, genetic analysis of human colonic adenomas and carcinomas from patients either with sporadic colorectal cancer or FPC commonly reveal mutations of the *APC* gene. Secondly, the recent finding that APC binds to  $\beta$ -catenin in cell adhesion complexes is interesting when characteristics of both normal and neoplastic cells are considered. Cell adhesion and cellular contacts are vital components of normal cell growth and signalling and are frequently disrupted in neoplasia. Finally, studies from the *min* mice show that mice with homozygous mutation of the murine *APC* gene are not viable and die *in utero*. These observations suggest an important role for APC in normal cell function, yet little is known of the cell biology of the APC protein especially in neoplastic cells. One way to address the question of the role of APC in neoplasia would be to introduce wild type protein into neoplastic cells. Previous experiments with this aim, transfected a whole chromosome 5 into colon cancer cells by microcell transfer. These microcell hybrid studies on two colon cell lines, SW480 and COKFu showed that introduction of chromosome 5 reduced tumorigenicity as assessed by subcutaneous injection of  $5 \times 10^6$ - $10^7$  cells into

athymic nude mice and also affected cellular morphology (Tanaka et al., 1991; Goyette et al., 1992). The addition of chromosome 5 to both colon cancer cell lines also resulted in an increase in cell doubling time and a decrease in growth in soft agar, both common markers of tumour cell aggressiveness *in vitro*. These chromosome transfer experiments however only suggest the presence of a suppressor gene on chromosome 5. In order to address whether the suppressor activity comes from the *APC* gene located on 5q21, a more precise method of analysis is required.

## 5.12 Gene Targeting

One way in which to determine whether *APC* mediates this response would be to introduce a normal copy of the gene into cancer cells. This could be achieved by the use of an *APC* transgene where the gene would be placed under the control of an exogenous promoter and transfected into cells. In view of its large size, however, *APC* is likely to transfer into target cells with a low efficiency. Moreover, transgenic strategies have the generic disadvantage of lack of control of expression of the gene once stably transfected. In general, transfection of genes under exogenous promoters leads to overexpression of the gene product which in the case of a suppressor protein may be sufficient to kill the cell. A better method for expressing potentially physiological levels of protein within a cell is gene targeting by homologous recombination.

Homologous recombination allows the exchange of genetic material between sequences of DNA that show sequence homology and was first exploited



experimentally in mammalian cells between two exogenous plasmid sequences in murine fibroblasts and rat cells (Folger et al., 1982). The exact mechanism of homologous recombination is still uncertain, but two major theories exist (Taken from Hooper 1992).

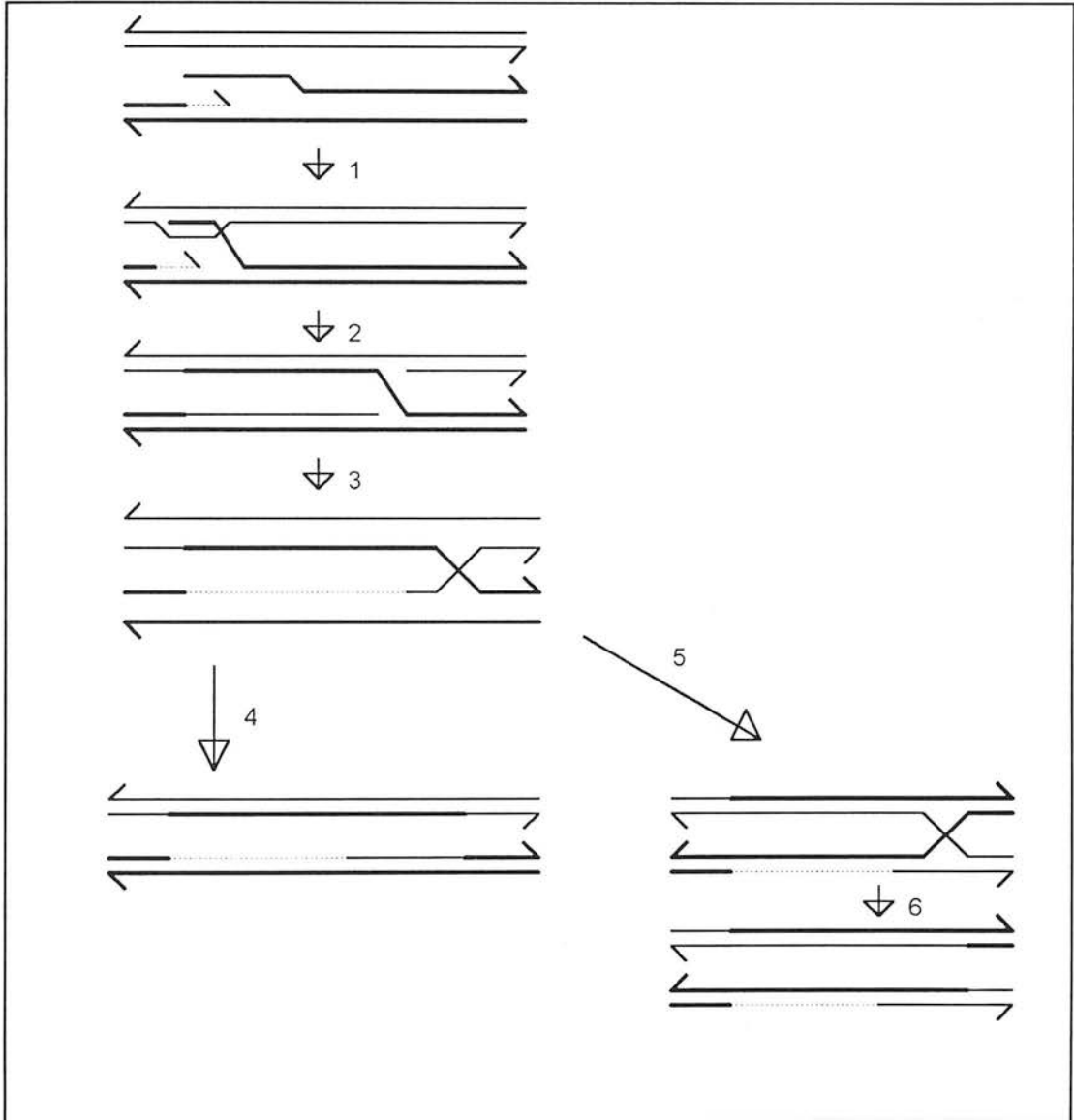


Figure 14. Meselson-Radding theory of homologous recombination.

In the first theory, a single strand break initiates recombination (Figure 14). The 3' end of the nick (3' DNA ends marked with a half arrow) acts as a primer for DNA repair synthesis displacing a single strand that invades the other homologue displacing a D-

loop (1). The D-loop is degraded and, by a combination of DNA synthesis on the donor and degradation on the recipient chromatid, an asymmetric heteroduplex is produced (2). Ligation (3), produces a Holliday junction which can diffuse along the molecules by a zipper-like branch migration mechanism. The Holliday junction may resolve by cutting the crossed strands (4) to give two double helical molecules each consisting of one parental strand and one strand that has undergone gene conversion without crossover. The newly synthesised DNA (dotted) has been copied from the donor homologue. Alternatively, the Holliday junction may undergo a process termed isomerisation in which the originally crossed strands become uncrossed and vice versa (5). This can then resolve by cutting the crossed strands (6) to produce crossover products also with a segment that has undergone gene conversion.

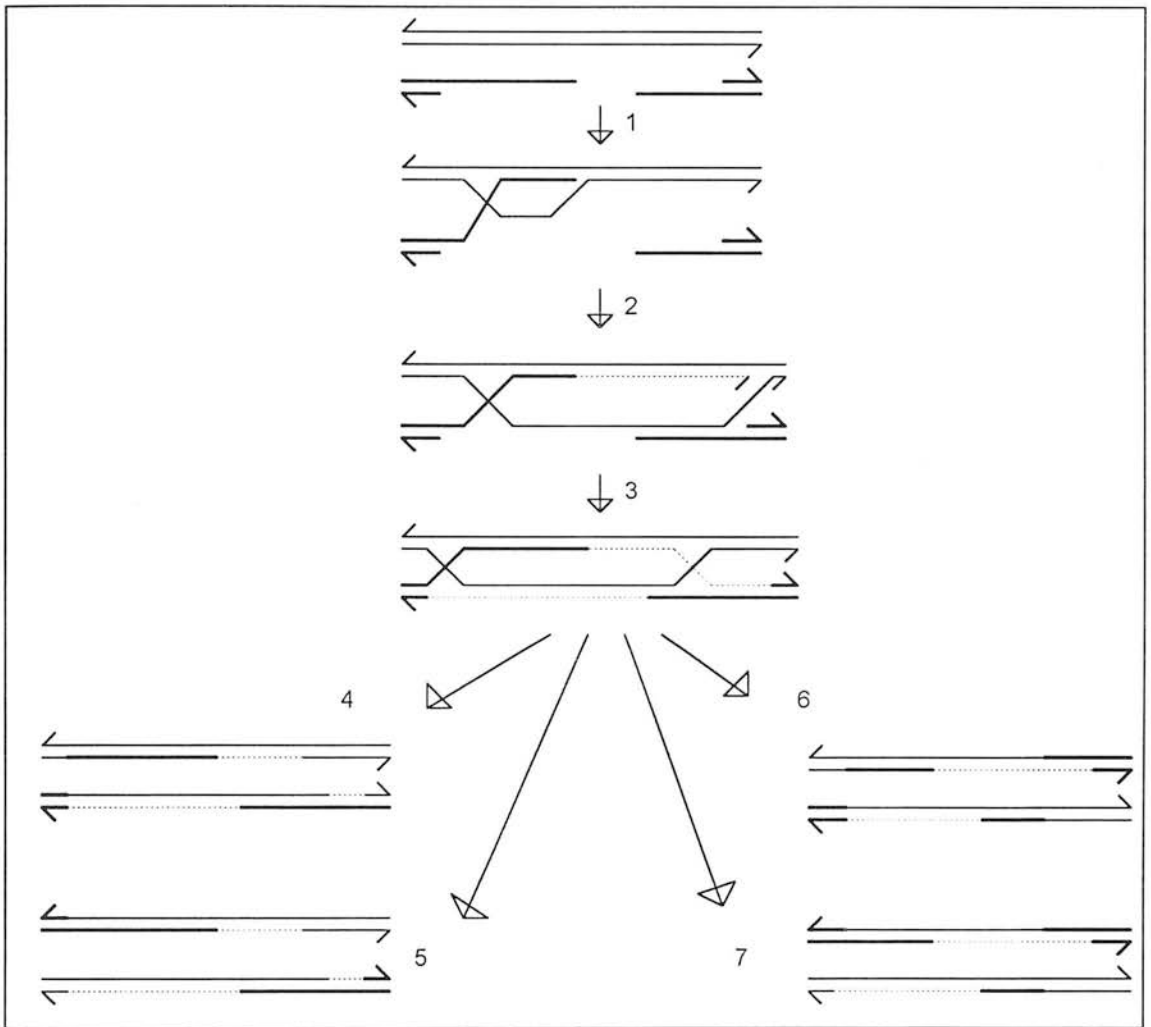


Figure 15 . Double strand break-repair model of recombination.

In the second model, recombination is initiated by a double strand cut in one duplex, followed by exonuclease attack to give a gap flanked by 3' single-stranded ends (Figure 15). One 3' invades the other duplex (1), displacing a D-loop enlarged by repair synthesis until the other 3' end can anneal to complementary single-stranded DNA (2). Repair synthesis from the second 3' end completes the gap repair, and ligation produces two Holliday junctions both of which can diffuse by branch migration (3). There are than four possibilities for resolution, depending upon whether isomerisation occurs at neither junction (4), the left junction only (5), the right

junction only (6), or both junctions (7). Resolutions (5) and (6) lead to crossover, whilst (4) and (7) do not.

In both models, the recombination machinery of the cell can mediate either conservative or non-conservative recombination which results in either the retention or loss of exogenous DNA respectively (Lin et al., 1984; Chakrabarti and Seidman 1986). Additionally, reciprocal or non-reciprocal recombination can occur with non-reciprocal homologous recombination being more common (Folger et al., 1985; Thomas et al., 1986). The frequency of recombination is dependent in an exponential manner on the length of homology that the two DNA sequences share and targeting using isogenic DNA has also been shown to increase the efficiency of homologous recombination over nonisogenic sequences (Hasty et al., 1991a; Deng and Capecchi, 1992). As well as homologous recombination, the DNA sequence can be incorporated into the endogenous DNA in a random fashion and mammalian cells preferentially mediate this non-homologous recombination or random integration (Figure 16)(Roth et al., 1985; Roth and Wilson, 1985). Random integration is not dependent on the stage of the cell cycle whereas homologous recombination occurs preferentially during the S-phase of the cell cycle (Wong and Capecchi, 1985; Wong and Capecchi, 1987). The common occurrence of random integration made essential the development of markers to select for correct homologous recombination events. This has previously been achieved either, by the incorporation of *hprt* (hypoxanthine phosphoribosyl transferase) minigenes into the targeting construct in such a way that when inactivated

## Figure 16. Positive-Negative Selection of Targeting events.

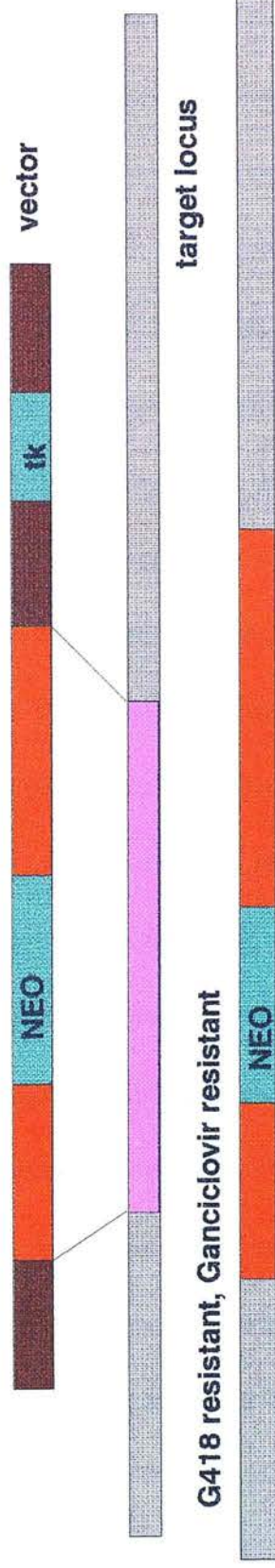
(Based on Capecchi 1989). Positive-Negative selection enriches for correctly targeted events. The *neo<sup>r</sup>* gene is inserted into the region of homology in such a way as to disrupt the gene sequence. The HSV-*tk* gene is linked to but outwith the region of homology.

Homologous recombination between the exogenous vector sequences and the endogenous gene will result in the disruption of the endogenous gene. Cells containing this event will be G418 resistant due to the presence of the *neo<sup>r</sup>* gene and ganciclovir resistant due to the lack of the HSV-*tk* gene.

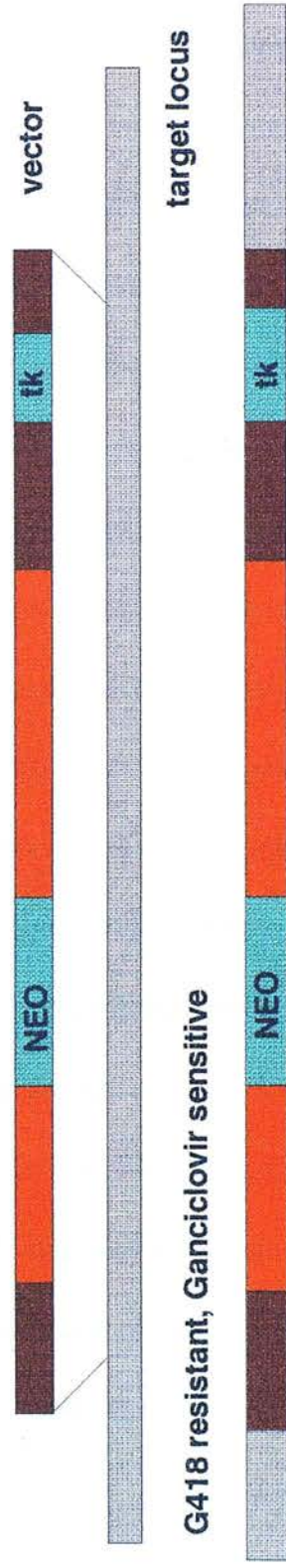
Non-homologous recombination or random integration occurring via the exogenous DNA ends results in the retention of the HSV-*tk* gene linked to the *neo<sup>r</sup>* gene. Such cells would be G418 resistant but ganciclovir sensitive.

Selectable marker genes are shown in blue. Regions of homology are shown in red for the exogenous vector sequences and pink for the endogenous gene respectively. Additional plasmid sequences are shown in brown and additional endogenous sequences are grey.

## Homologous Recombination



## Random Integration



cells will grow in the base analogue 6 thioguanine which kills cells with an active *hprt* gene. Or, by the functional replacement of an *hprt* gene by homologous recombination allowing selection in HAT (hypoxanthine, aminopterin, thymidine) medium (reviewed in Capecchi 1989). Another common method for selection of correctly targeted events is the introduction of drug resistance markers with the homologous DNA (Folger et al., 1984; Smithies et al., 1984). Examples of this are the bacterial neomycin resistance gene (*neo*) which in mammalian cells confers resistance to the drug G418, and the Herpes Simplex Virus thymidine kinase gene (*HSV-tk*) which confers susceptibility to the nucleoside analogue ganciclovir. The introduction of the *neo* gene into the region of homology of the exogenous DNA will allow the selection of homologous recombination events and will disrupt the target gene thereby creating a null allele. Positive/negative selection (PNS) allows enrichment for homologous recombination events. This is achieved by the incorporation of the *neo* resistance gene into the region of homology and the *HSV-tk* gene outside this region within a plasmid vector. During homologous recombination, the *neo* gene will be transferred with the region of homology, whereas the *HSV-tk* gene will not be transferred resulting in a selection for correct events in both G418 and ganciclovir. Non-homologous recombination and random insertions occur via the DNA ends and normally involve the introduction of the entire sequence, resulting in the incorporation of the *HSV-tk* gene and subsequent sensitivity to ganciclovir (Thomas et al., 1986; Mansour et al., 1988)

There are two common types of targeting vector, replacement and insertion vectors (Figure 17). As suggested by the name, replacement vectors replace the endogenous DNA with the exogenous sequence. In contrast, insertion vectors insert the entire vector sequence into the homologous locus. Some studies have suggested that insertion vectors are more efficient than replacement vectors at mediating homologous recombination (Hasty et al., 1991b), whereas others have detected no difference in the targeting efficiency between the two types of vector (Thomas and Capecchi, 1987).

The majority of gene targeting to date has involved targeting into murine embryonic stem (ES) cells. Murine ES cells were first isolated by Evans and Kaufman, and Martin in 1981. These cells are pluripotent and capable of contributing to the germline of animals created by the introduction of ES cells into host blastocysts. This has allowed the targeting of ES cells by transfection of modified DNA sequences *in vitro* by standard molecular techniques. The ES cells are then selected for homologous recombination events as described above. Cells with a correct homologous recombination event are then reintroduced into a cultured host blastocyst by microinjection and the blastocyst is then replaced in a pseudo-pregnant female mouse which may or may not generate germline chimeras. Coat colour markers of host and recipient strains allow the distinction of the chimeras in which the ES cells have contributed and mating of the offspring of germline chimeras and wild type crosses



## Figure 17. Gene disruption by replacement and insertion targeting vectors.

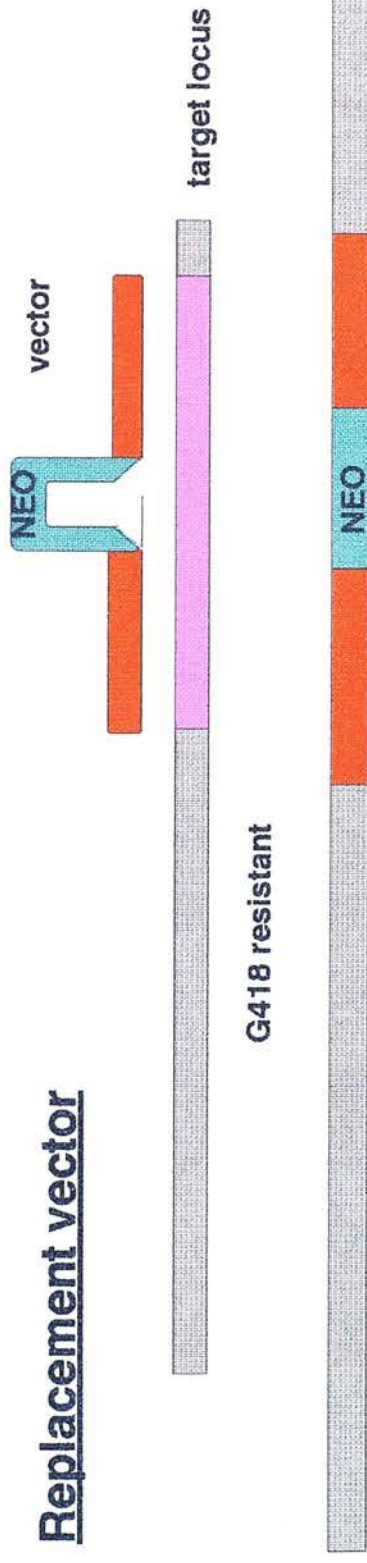
(Based on Capecchi 1989)

A sequence replacement vector contains the *neo<sup>r</sup>* gene within the gene to be targeted. Homologous recombination between the regions of homology replaces the genomic sequence with the vector sequence containing the *neo<sup>r</sup>* gene and therefore rendering the cells resistant to G418 whilst disrupting the gene of interest.

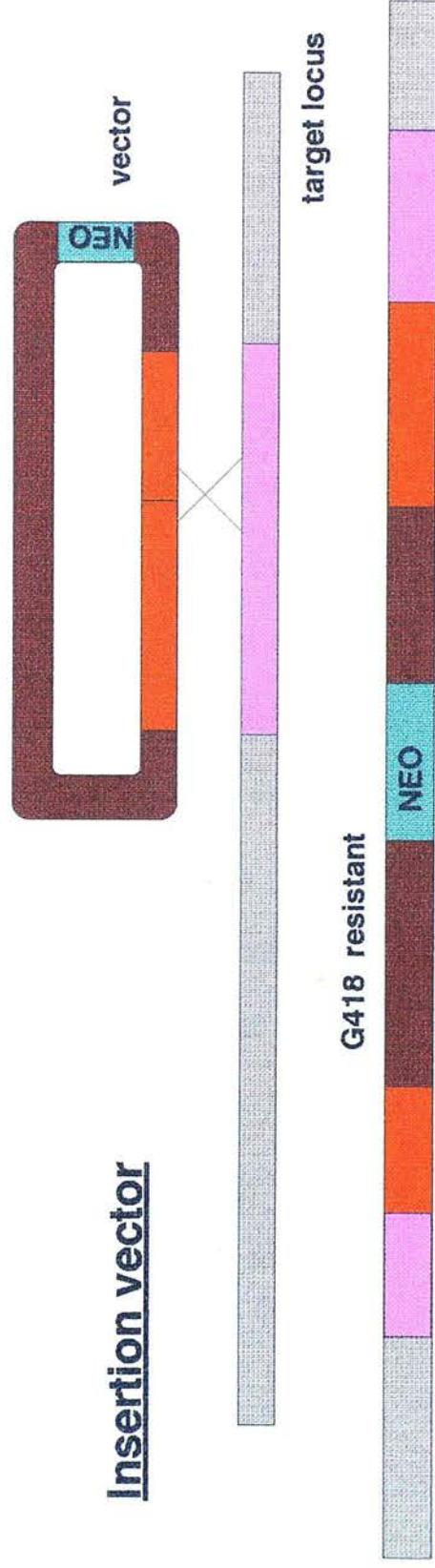
Sequence insertion vectors are designed such that linearisation within the region of homology places vector ends adjacent to each other. The pairing of these ends with the endogenous sequences and subsequent recombination results in the complete insertion of the vector producing a duplication of the region of homology. The presence of the *neo<sup>r</sup>* gene in the vector again confers resistance to G418.

Selectable marker genes are shown in blue. Regions of homology are shown in red for the exogenous vector sequences and pink for the endogenous gene respectively. Additional plasmid sequences are shown in brown and additional endogenous sequences are grey.

## Replacement vector



## Insertion vector



allows for the identification of homozygous animals (Bradley et al., 1984; reviewed in Capecchi 1989).

This approach has been particularly effective in the study of genes commonly involved in human disease such as the cystic fibrosis (*CF*), retinoblastoma (*Rb-1*) and *p53* genes (Dorin et al., 1992; Lee et al., 1992; Jacks et al., 1992; Clarke et al., 1992; Donehower et al., 1992). The production of null alleles provides valuable data on gene function. However, a large number of genes are modified physiologically by more subtle alterations to their sequence such as base pair changes, for example activation of the *K-ras* oncogene and inactivation (or gain of function) of tumour suppressor genes such as *APC* and *p53*. Other targeting strategies have therefore been devised to allow more subtle modifications of the genome to occur. The "hit and run" (alternatively called "in-out") strategy generates such cells without the permanent incorporation of selectable markers (Valancius and Smithies 1991; Hasty et al., 1991c). Hit and run targeting vectors contain a region of homology, and selectable markers such as *neo* and *HSV-tk* within the plasmid backbone (Figure 18). The vector is linearised within the region of homology and in a single reciprocal recombination event the entire vector inserts into the locus generating a linear duplication of the homologous regions separated by selectable marker sequences. Selection in G418 will identify cells that have incorporated the vector containing the *neo* resistance gene. Identification of correct homologous recombination events is then achieved by isolation of DNA from these cells and a variety of screening methods commonly based

## Figure 18. Hit and Run Gene Targeting.

(Based on Hasty et al., 1991c).

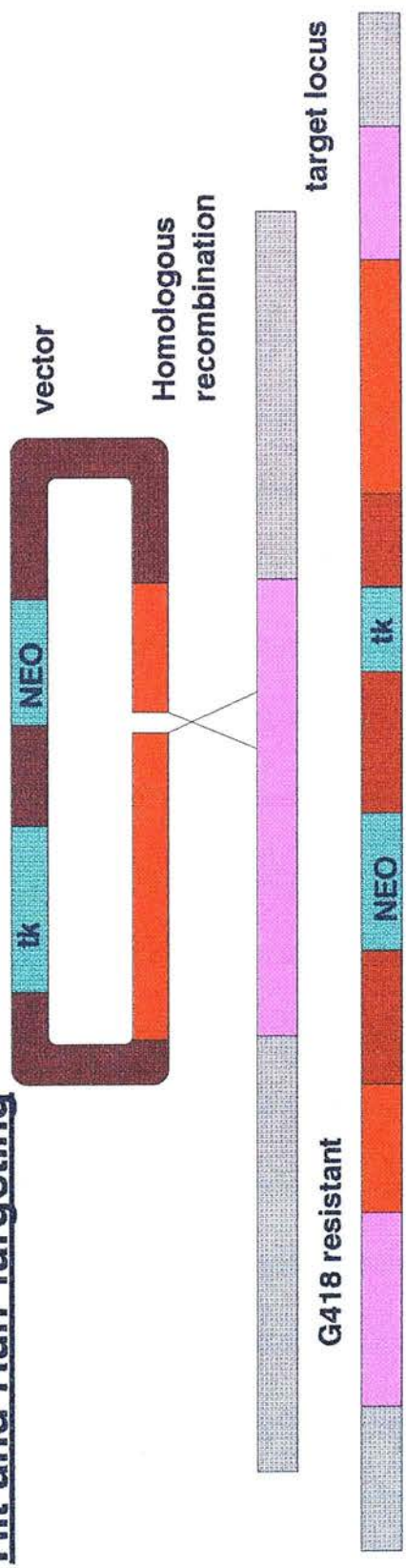
Hit and run targeting allows a small mutation to be introduced into a nonselectable gene in a two step recombination procedure.

The first step involves a single recombination event between the target gene and the linearised insertion vector. The vector contains the small required alteration within the target gene (\*) as well as both the *neo<sup>r</sup>* and HSV-*tk* genes. The entire vector inserts into the target locus creating a linear duplication of the genomic sequences. The cells can be selected in G418. G418<sup>r</sup> clones of cells are screened to detect those in which a correct recombination event has occurred.

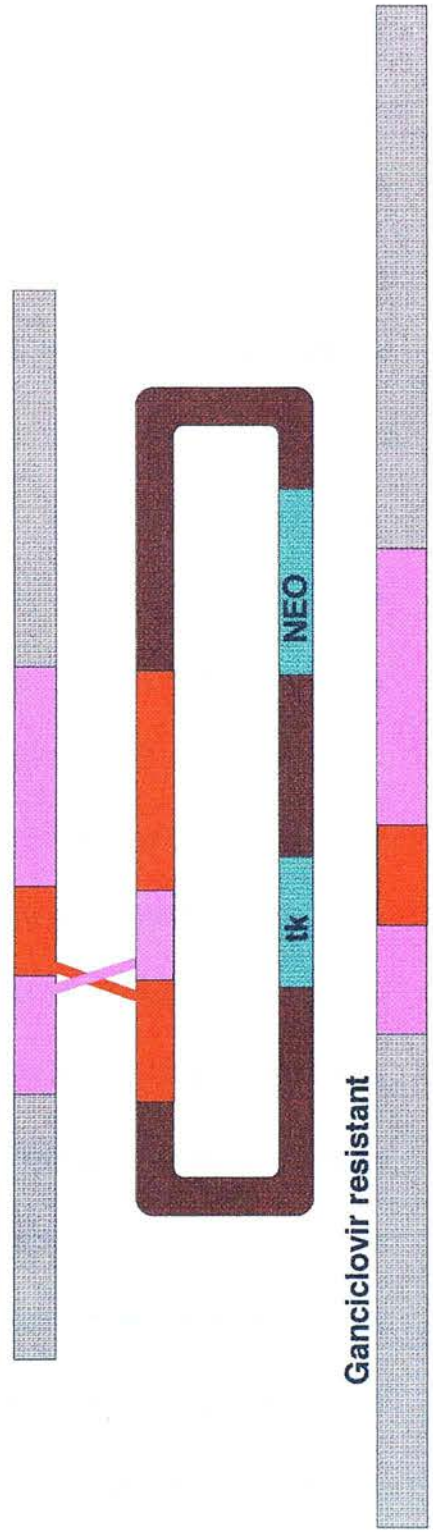
The second step involves an intrachromosomal recombination event within the duplication. The plasmid, *neo<sup>r</sup>*, HSV-*tk* and the duplication from either vector or genomic origin are lost depending on the site of the recombination event. Cells can then be selected in ganciclovir due to the loss of the HSV-*tk* gene. Ganciclovir resistant clones can then be screened for the presence of the mutation.

Selectable marker genes are shown in blue. Regions of homology are shown in red for the exogenous vector sequences and pink for the endogenous gene respectively. Additional plasmid sequences are shown in brown and additional endogenous sequences are grey.

# Hit and Run Targeting



## Intrachromosomal recombination



on the generation of characteristic PCR fragments, or the use of radiolabelled DNA probes after Southern analysis to identify fragments unique to the correct event. Cells containing an homologous recombination event are then grown in the presence of ganciclovir which selects for a second reciprocal intrachromosomal recombination event and the loss of the *tk* gene, which depending on the exact site of the crossover removes the *neo* and *tk* sequences and one copy of the homologous region. If the cross over occurs before the sequence change, then the introduced change will be retained and the endogenous sequence lost.

The fact that most targeting has been done in ES cells means that little is known about the targeting frequency in non-ES cells such as cancer cells. One study of gene targeting in somatic cells disrupted an activated *K-ras* gene in two colon carcinoma cell lines. Morphological and biological changes such as a decrease in cell growth both *in vitro* and *in vivo* were noted in targeted cells (Shirasawa et al., 1993). A significant problem with corrective targeting of a suppressor such as *APC* in cancer cells may be that production of wild type protein may be sufficient to kill the cell. The chromosome transfer experiments described earlier, however, suggest that the presence of wild type APC protein is compatible with cell growth *in vitro* at least in the two colon cancer cell lines studied. Targeting of the *APC* gene will address the question of whether correction of an "early" event in tumorigenesis will have any effect on cell lines which have already accumulated additional genetic lesions normally associated with the later stages of tumour progression

Many colon carcinoma cell lines exist, and their study has provided valuable information on the progression of colorectal disease (Paraskeva et al., 1984; McBain et al., 1984). One of the best characterised colon cell lines is SW480.

### 5.13 The SW480 cell line

SW480 was established from a Dukes B stage adenocarcinoma removed from a 50 year old Caucasian male (Leibovitz et al., 1976). Published karyotypes of SW480 vary between studies. One such karyotype suggests that SW480 has lost a copy of each of chromosomes 5 and 18 and also has a derived chromosome consisting of a reciprocal translocation between (5;20)(q15;p11) (Yaseen et al., 1990). Another study suggests that there is also an additional chromosome 17 and 2-4 copies of the derived 5;20 translocation (Tomita et al., 1992). Studies on particular genes have shown an activating point mutation of *K-ras* in codon 12 (Gly-Val) (Capon et al., 1983), amplification of *c-myc* (Suarez et al., 1987), point mutations of *p53* in codons 273 (Arg-His) and 309 (Pro-Ser) (Nigro et al., 1989), deletion and low expression of *DCC* (Yaseen et al., 1990; Goyette et al., 1992), and finally loss of the normal allele of *APC* with a point mutation at codon 1338 (Gln-stop) in the remaining allele (Nishisho et al., 1991). Tumorigenicity was originally assessed by injection of  $10^6$  cells into athymic nude mice either into the footpad or subcutaneously (Trainer et al., 1988). Only the cells introduced into the footpad produced tumours, appearing approximately 21 days after injection, whilst a more recent study in which  $10^7$  cells



injected subcutaneously produced tumours within 7 days (Goyette et al., 1992). Under optimal growth conditions *in vitro* the cell line has a doubling time of around 22 hours. The cells weakly express keratin and have microvilli, tight and adherens junctions and desmosomes (Trainer et al., 1988).



## **5.2 Experimental Design**

The basic strategy proposed involves the generation of 'hit and run' insertion targeting vectors incorporating approximately 3Kb of exon 15 of the *APC* gene, homologous to the region spanning the truncating mutation at codon 1338 found in SW480 cells. Correction of the point mutation should restore a PstI restriction enzyme site in the cells which would be identifiable by either Southern analysis or PCR-based technologies involving PstI restriction digestion. An exonic region of homology is generated by PCR amplification of "normal" genomic DNA and therefore expected to behave more like isogenic than non-isogenic DNA which has been shown to have a lower absolute targeting frequency. The vector also contains suitable selectable markers. The use of the *neo* resistance gene allows selection of a first recombination event with the resulting insertion of the entire vector sequence. Analysis of the G418 resistant clones is by Southern blotting after i.)PstI and ii.)StuI restriction enzyme digestion and subsequent radiolabelled probing with a probe that spans the insertion point of the vector into the endogenous DNA to identify a correct insertion event (Figure 19). The restoration of a PstI restriction site allows identification of both the endogenous mutant and exogenous wild type fragments created by the linear duplication of the homologous sequences thereby eliminating clones that have undergone branch migration events leading to duplication of either endogenous or exogenous sequences. Incorporation of the *HSV-tk* gene into the vector allows selection for a second intrachromosomal recombination event. Correct clones are grown and selected in ganciclovir to select for a recombination event in which the

**Figure 19. The Hit and Run protocol for corrective targeting of the SW480 cell line (1).**

Linearised pPNT.APC or pPNT.APCwt inserts into the exon 15 APC locus as previously described (Figure 16). (pPNT.APC and pPNT.APCwt are assumed to be identical except for the presence of a truncating mutation (M) at codon 1359 in pPNT.APC).

The resulting linear duplication of the loci and subsequent resistance to G418 allows screening for a correct insertion event by Southern analysis after a StuI restriction enzyme digest using the 2kb APC probe pAPC.KC. Correct insertion should reveal a unique 5.3kb band.

**N**-NsiI restriction enzyme site.

**P**-PstI restriction enzyme site.

**P\***-PstI site mutated in the SW480 cell line at codon 1338 replaced in targeting vectors pPNT.APC and pPNT.APCwt.

**E**-EcoRI restriction enzyme site.

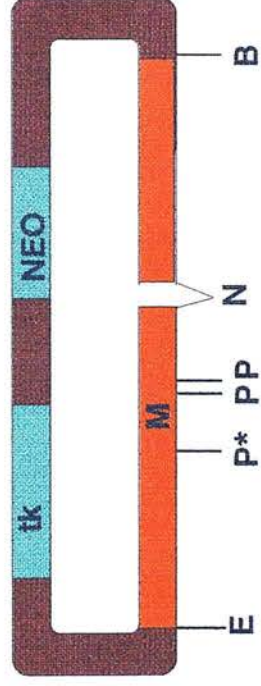
**B**-BamHI restriction enzyme site.

**S**- StuI restriction enzyme site.

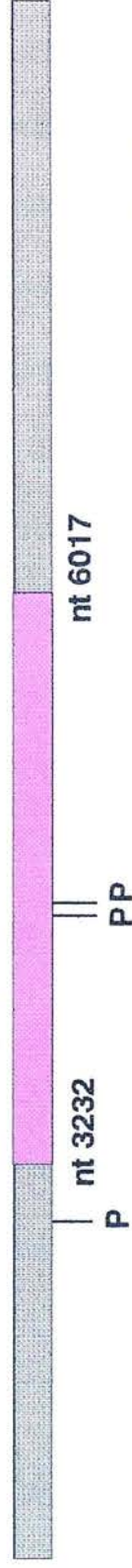
**M**-novel truncating mutation at codon 1359 in vector pPNT.APC

Selectable marker genes are shown in blue. Regions of homology are shown in red for the exogenous vector sequences and pink for the endogenous gene respectively. Additional plasmid sequences are shown in brown and additional endogenous sequences are grey.

# pPNT.APC



## SW480 APC exon 15



Homologous recombination

G418 resistant

2kb probe

2kb probe



5.3kb

sequence is resolved with the loss of the *HSV-tk* gene and subsequent resistance to the toxic effects of ganciclovir (Figure 20). Ganciclovir resistant clones are analysed to establish whether the resulting endogenous sequence has resolved back to the original mutation at codon 1338 or whether correction of the mutation has occurred. This depends upon the location of the second cross-over event. If this event is on the same side of the mutation to the first crossover (not necessarily where the vector linearises due to branch migration) the resulting sequence will contain the original mutation. If however the cross-over occurs after codon 1338 then the mutation will be corrected. This is detectable by Southern digest after PstI digest and probing with a suitable *APC* probe. Correction of the codon 1338 mutation will restore a PstI site with characteristic band fragment size. This analysis should also reveal the codon 1338 mutant fragment bands due to the two other copies of *APC* present. As the results of a correct targeting event of *APC* in SW480 cells were unknown, it was necessary to control the entire experiment by replacing the mutation in SW480 with another mutation elsewhere in the *APC* sequence. This would provide a control for the targeting itself without the risk of introducing suppressor protein which may for reasons previously discussed result in the production of no viable clones. In the course of making the correcting targeting vector a suitable mutated vector was derived.

## **Figure 20. The Hit and Run protocol for corrective targeting of the SW480 cell line (2).**

The second intrachromosomal recombination event results in either a reversion to the original mutated sequence in SW480 or the correction of the mutation as previously described (Figure 16).

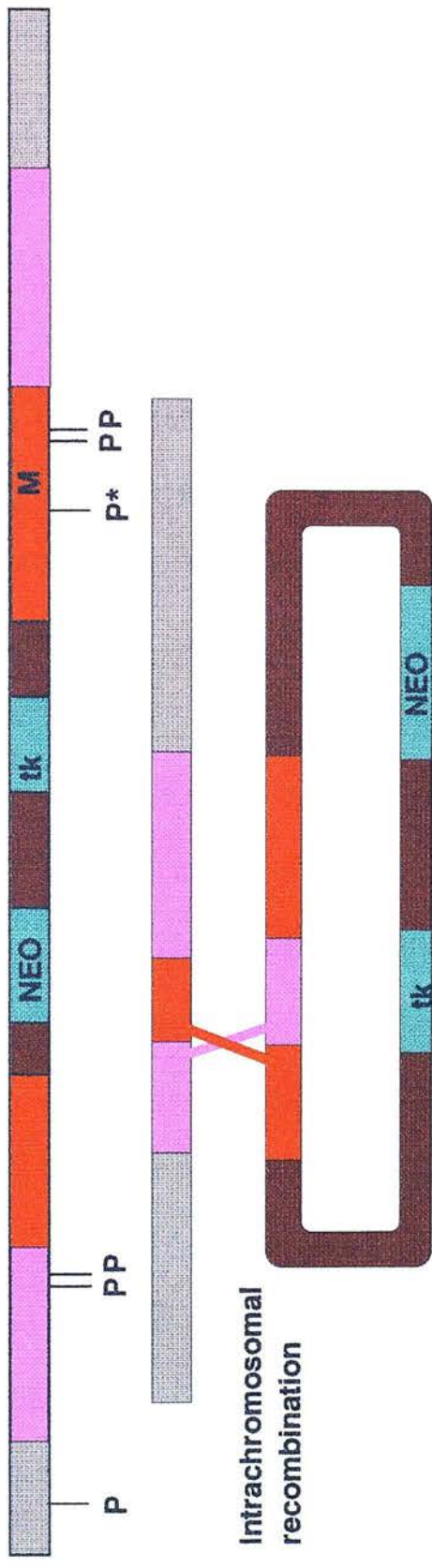
Screening of ganciclovir resistant colonies by Southern analysis of PstI restriction digested DNA, probed with the 2kb APC probe pAPC.KC should reveal bands of 1.4kb and 0.4 kb in corrected clones. The 1.8kb band previously seen (Figure 19) will be evident in uncorrected SW480 clones.

**P**-PstI restriction enzyme site.

**P\***-PstI site mutated in the SW480 cell line at codon 1338 replaced in targeting vector pPNT.APC and corrected SW480 clones.

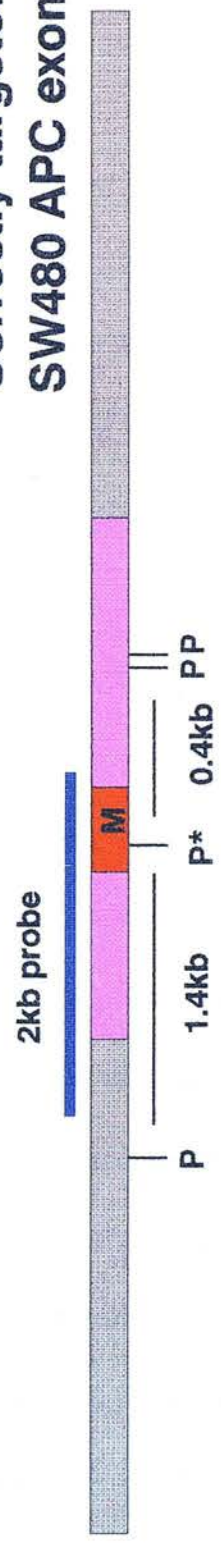
**M**-novel truncating mutation at codon 1359

Selectable marker genes are shown in blue. Regions of homology are shown in red for the exogenous vector sequences and pink for the endogenous gene respectively. Additional plasmid sequences are shown in brown and additional endogenous sequences are grey.

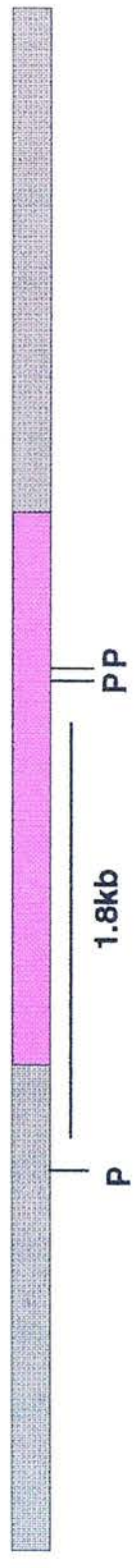


Ganciclovir resistant

Correctly targeted  
SW480 APC exon 15



SW480 APC exon 15



## **5.3 Results**

### **5.31 Properties of the SW480 cell line**

SW480 DNA was digested with the restriction enzyme PstI and after Southern transfer and probed with a radiolabelled genomic PCR product of 2Kb to a region of exon 15 of APC (nucleotides 2480-4498) spanning the codon 1338 mutation site. The size of the fragments observed indicated that these cells do not possess a wild type *APC* gene, only *APC* DNA with the codon 1338 mutation and hence without a PstI site at codon 1338 was present in the cell line (Figure 21).

Metaphase chromosome spreads were prepared for fluorescent in-situ hybridisation (FISH) studies. A small number of metaphase spreads were counted for chromosome number; the number of chromosomes observed (50-60) was within the range reported in other studies (Yaseen et al., 1990; Tomita et al., 1992). Fluorescent in-situ hybridisation (FISH) using probes to the 5q21 region and to the *APC* gene revealed 3 copies of 5q21. Chromosomal locations of the three copies of the gene were identified by chromosome paints. One was shown to be a single copy of chromosome 5, whilst the other two gene copies appeared to reside on derived chromosomes t(5;20)(Figure 22). This translocation is probably the t(5;20)(q15;p11) already reported (Yaseen et al., 1990). It is not known whether the derived/translocated chromosome copies of *APC* are expressed.

**Figure 21. Southern Analysis of exon 15 of the *APC* tumour suppressor gene in SW480 and tumour genomic DNA samples.**

Lane 1 SW480 genomic DNA digested with the restriction enzyme PstI and probed with the 2kb *APC* probe pAPC.KC.

Lane 2 Tumour sample X genomic DNA digested with the restriction enzyme PstI and probed with the 2kb *APC* probe pAPC.KC.

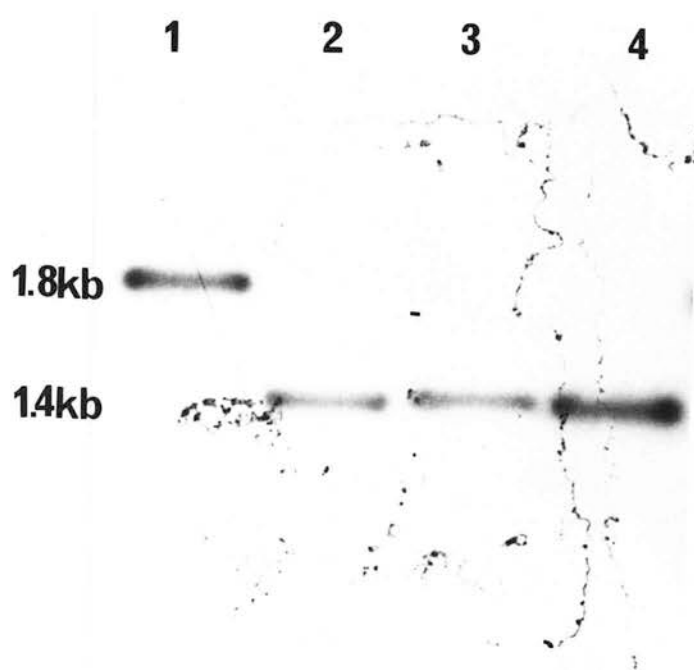
Lane 3 Tumour sample Y genomic DNA digested with the restriction enzyme PstI and probed with the 2kb *APC* probe pAPC.KC.

Lane 4 Tumour sample Z genomic DNA digested with the restriction enzyme PstI and probed with the 2kb *APC* probe pAPC.KC.

Tumours X,Y and Z were randomly selected from the sporadic colorectal tumour bank (Dept. Pathology, Edinburgh).

The SW480 digest in lane 1 shows a band of approximately 1.8kb compared to the three tumour samples in lanes 2, 3 and 4 which reveal bands of 1.4kb. This indicates the loss of a PstI restriction enzyme site at codon 1338.





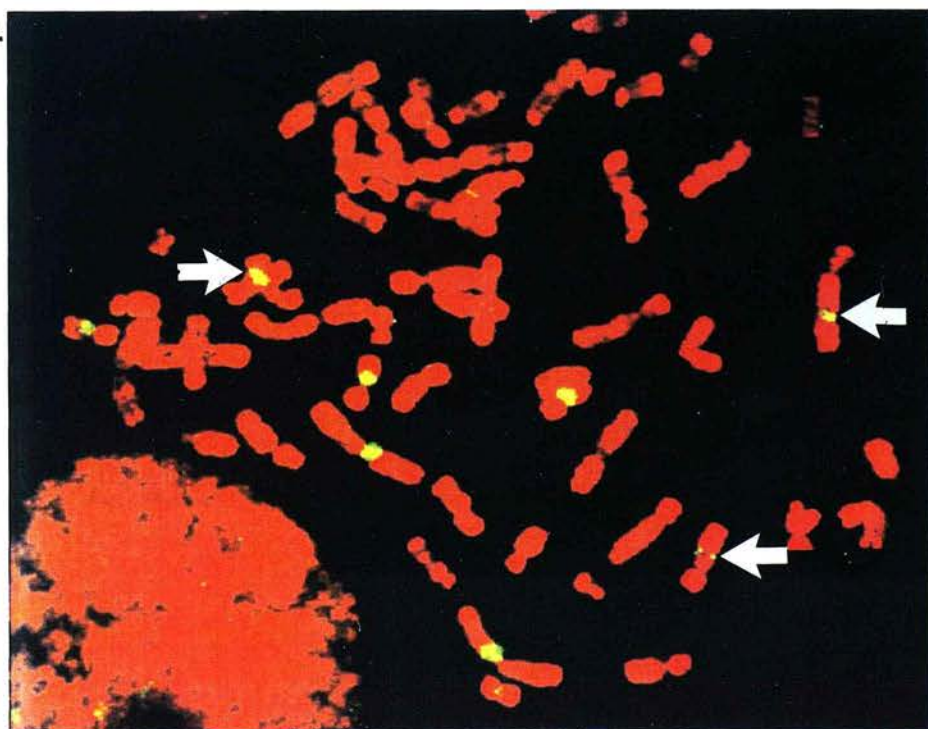
**Figure 22. Fluorescent *in situ* Hybridisation (FISH) on the SW480 cell line.**

- a.) Chromosome painting of SW480 metaphase spreads. Chromosome 5 sequences in red are detected by a Texas Red labelled chromosome paint, and chromosome 20 sequences by a fluoresceine isothiocyanate (FITC) labelled chromosome 20 paint in green. This shows 1 copy of chromosome 5, 2 copies of chromosome 20, 2 derived chromosome 5(5;20)(q15;p11) and 2 derived chromosome 20(5;20)(q15;p11) reciprocal translocations.
- b.) FISH analysis of metaphase spread of SW480 cells using an *APC* YAC probe labelled with FITC (green). Chromosomes are counterstained with propidium iodide (PI)(red). The YAC binds to the centromeres of chromosomes 1 and 16 by unknown sequences. The *APC* gene sequences are identifiable on the remaining copy of chromosome 5 and also on the 2 derived chromosome 20 translocated chromosomes. (Indicated by arrows).
- c.) FISH analysis of metaphase spread of SW480 cells using an *APC* cosmid probe labelled with Texas Red (red). Chromosome 20 sequences are labelled green with FITC and chromosomes are counterstained blue with DAPI. This confirms the location of *APC* sequence on the one copy of chromosome 5 and the derived 20 translocation chromosomes.

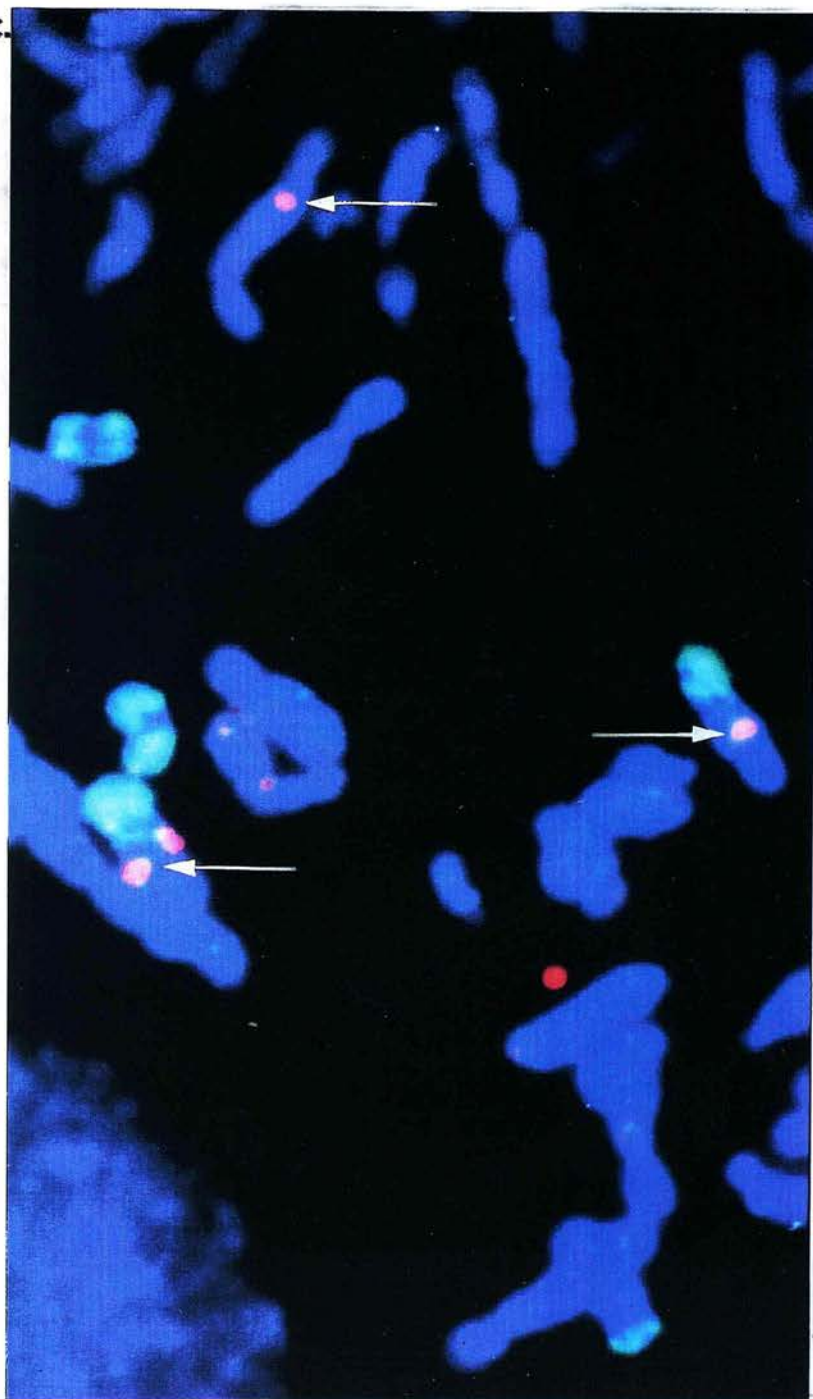
a.



b.



C.



Tumorigenicity was assessed by subcutaneous injection of  $10^7$  SW480 cells into SCID mice maintained in incubators. Tumours were visible in all animals three weeks post-injection. Histological examination of these tumours revealed characteristic poorly differentiated anaplastic carcinoma (Figure 23).

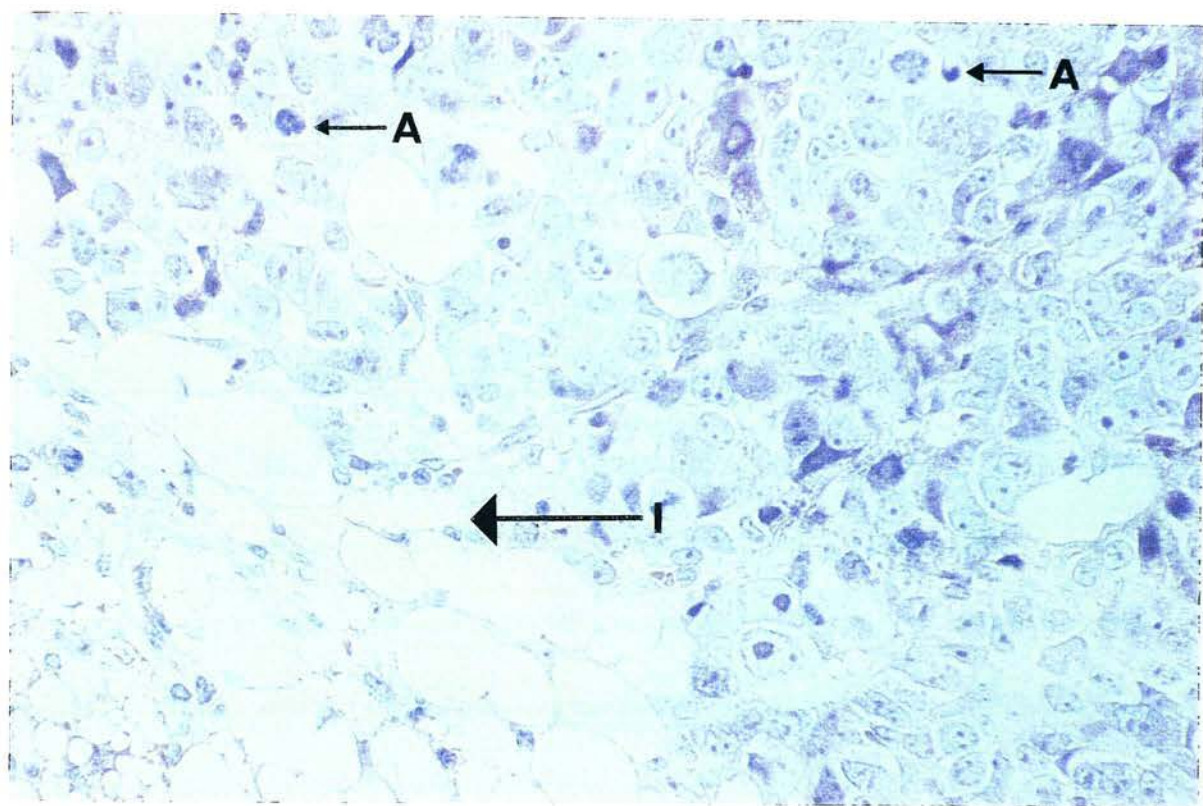
### 5.32 Construction of Targeting Vectors

A "hit and run" targeting vector pPNT.APC, was constructed by the cloning of a 2.8Kb PCR fragment of the *APC* gene exon 15 (nucleotides 3232-6017) into the plasmid pPNT. The source DNA was morphologically normal mucosa from a patient with a sporadic colorectal carcinoma. PCR primers were designed to incorporate different restriction enzyme sites (BamHI and EcoRI) into the ends of the PCR product. Digestion of the pPNT parental vector with the same restriction enzymes allowed directional cloning of the product into pPNT to generate the vector pPNT.APC. Restriction mapping of the generated plasmid using a variety of restriction endonucleases confirmed that pPNT.APC was as shown (Figure 19). In order to confirm the suitability of this vector for corrective targeting, it was essential to confirm that the entire 2.8Kb *APC* fragment was of the wild type sequence. In order to sequence this relatively large fragment of DNA it was excised from the pPNT vector using BamHI and EcoRI restriction digests and cloned into the pGEM7Zf+ plasmid as described previously. This vector is designed to allow the generation of nested deletions of the fragment to be sequenced in the Erase-a-Base system.

**Figure 23. Haematoxylin and Eosin stained xenograft tumour**

Highly anaplastic carcinoma removed from a SCID mouse two weeks post subcutaneous injection of  $10^7$  SW480 cells and stained with haematoxylin and eosin, showing invasion of the fat layer (I) and characteristic apoptotic bodies (A).

(Approximately x200)





Unexpectedly, sequence analysis of the entire 2.8Kb APC fragment in pPNT.APC revealed the existence of a truncating mutation in codon 1359 (AAA-TAA) (Figure 24). Reasons for this will be discussed later.

In order to generate a targeting vector with a wild type *APC* sequence another vector pPNT.APCwt (Figure 19), was established by the same method except, PCR amplification of the 2.8Kb fragment was from the genomic DNA of an individual free from colorectal cancer. pPNT.APCwt remains to be sequenced.

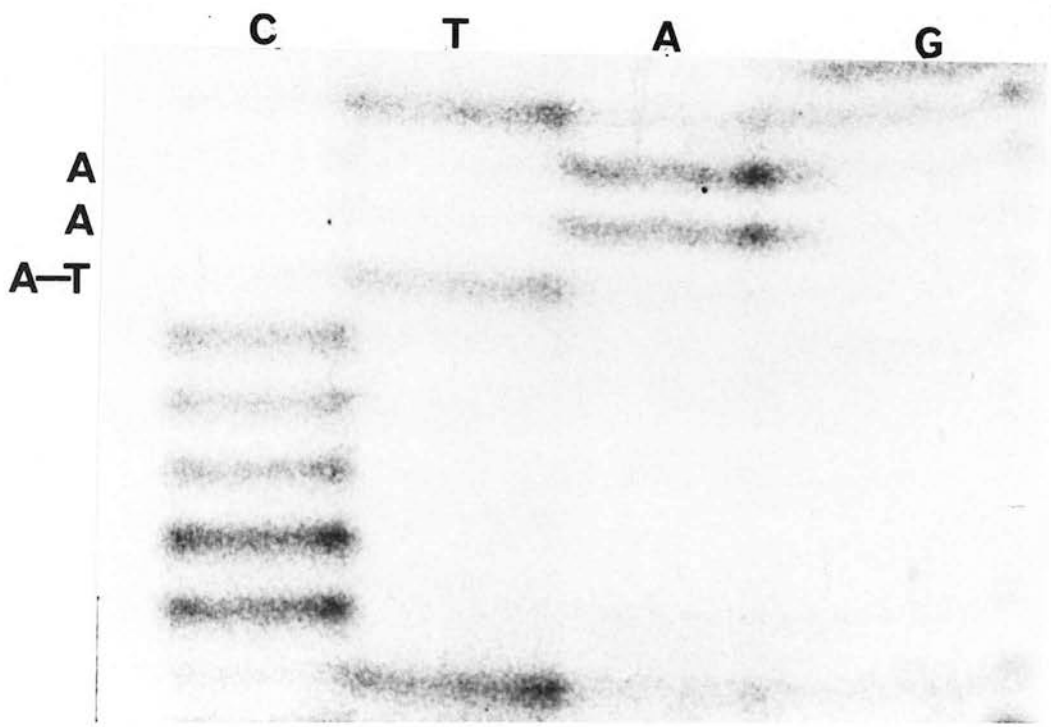
### 5.33 G418 resistant clones and analysis

The effect of introduction of a wild type APC gene into SW480 is uncertain. It is possible that clones containing wild type APC protein may be either not viable or at a growth disadvantage compared with non-corrected clones. In order to assess the efficiency of targeting into SW480 cells without producing a corrected APC protein, cells were transfected with pPNT.APC, the vector containing the novel mutation. Electroporation conditions were optimised and 60µg of linearised pPNT.APC was electroporated into  $10^8$  SW480 cells. The concentration of G418 for selection was established by analysing  $10^4$  SW480 cells in varying concentrations of G418 sulphate. Cells grown in 0, 200 or 400 µg/ml G418 were viable and confluent after 5 days. In contrast, cells grown in 600, 800, or 1000µg/ml were all dead by 5 days. 600µg/ml G418 was therefore chosen for selection. These results also suggested that the chance of a cell surviving selection without a transfected *neo* resistance gene is less than 1 in



**Figure 24. Sequence analysis of the pPNT.APC targeting vector revealing a truncating mutation in codon 1359, exon 15 of the *APC* tumour suppressor gene.**

Sequence analysis of the 2.8kb *APC* exon 15 fragment in pPNT.APC. A truncating mutation AAA-TAA (Lysine to Stop) was identified at codon 1359.



$10^4$ . After cell plating at densities of  $10^7$  and  $5 \times 10^6$  and subsequent selection in G418, approximately 300 G418 resistant clones were picked and plated into 24 well plates. 211/300 clones grew to confluence and were harvested. Analysis of these G418 resistant clones remains to be done.

## **5.4 Discussion**

There is much evidence to support the idea that a gene important in the maintenance of a tumorigenic phenotype in some colon cancer cell lines resides on chromosome 5q21. Studies in which the entire chromosome 5 was replaced into the colon cancer cell lines SW480 and COKFu by microcell transfer revealed a substantial reduction in the tumorigenicity in the two lines (Goyette et al., 1992; Tanaka et al., 1991). In order to establish whether this response is mediated by the *APC* gene this study attempted to correct specifically the truncating mutation present at codon 1338 in the *APC* gene in the SW480 cell line. Characterisation of the SW480 cell line by FISH revealed a potential three copies of the *APC* gene, whilst restriction digestion with the restriction endonuclease PstI detected only *APC* sequence with the codon 1338 mutation.

SW480 was considered a suitable cell line for these experiments for a number of reasons. The first of these is that it is relatively well characterised with regard to genetic abnormalities associated with colorectal cancer. However, there is confusion in the literature as to the number of copies of *APC* contained in the SW480 cell line. From the FISH studies presented here it appears that there are three copies of the gene. This is in contrast to the microcell transfer experiments on SW480 which stated that only one copy of the *APC* gene was present (Goyette et al., 1992). The reasons for the discrepancies are unknown however and may be due to variability between SW480 subclones. However, three copies of *APC* have been confirmed in additional

SW480 subclones from other laboratories and it is possible that the SW480 subclone used in the microcell transfer experiments may contain up to five copies of the mutant *APC* gene (J. Groden and A. Farmer Pers. Comm.) It is important to know the number of potential targets for incorporation of the vector in this cell line so that potential gene dosage and dominant/negative effects can be assessed. As previously mentioned, it is possible that the potential reduction in tumorigenic properties may place corrected cells at a growth disadvantage compared to cells containing only mutant *APC* and therefore corrected cells may not be identifiable. This outcome would be indistinguishable from one in which homologous recombination had not occurred. In order to assess the frequency with which homologous recombination occurs at this locus with the virtually identical targeting vectors, transfection with the pPNT.APC vector should allow correction of the codon 1338 mutation and introduction of a novel truncating mutation downstream at codon 1359. This will allow the targeting frequency to be assessed whilst still retaining mutant *APC* which should retain the tumorigenic phenotype of the parental SW480 cells. A further important monitor of the success of corrective targeting would be Western analysis using N-terminal antibodies to the APC protein. Antibodies to the APC protein are currently being made in this laboratory.

If correction of the *APC* gene by pPNT.APCwt occurs and wild type protein is translated and results in viable cells, then by extrapolation from the microcell transfer experiments, a reduction in tumorigenicity might be expected. This could be assessed

by common biological assays such as doubling times, growth in soft agar and tumorigenicity in SCID mice compared to the parental SW480 cell line.

SW480 is also a desirable cell line in which to correct one genetic lesion involved in colorectal tumour progression because this can be taken in the context of the other known genetic abnormalities within the cell line. *APC* mutation is generally considered to be an early genetic event in colorectal tumorigenesis and it is of interest to consider the effect correction of an early event in an already highly malignant cell line may have. It should be noted that other colorectal cell lines may involve other pathways to progression. For example, the VACO 235 and its derived clone 411 colon cancer cell lines contain mutant *ras* protein, mutant *APC* protein, express no *DCC* transcripts and have only wild type *p53* protein, in contrast to the SW480 cell line which contains two mutations of the *p53* gene (Markowitz et al., 1994; Goyette et al., 1992). VACO 235 is non-tumorigenic despite the genetic lesions affected, whilst the grand daughter cell line VACO 411 is tumorigenic in nude mice without acquiring any additional lesions in commonly affected genes including *p53*. This suggests that mutant *p53* is not necessarily required for a tumorigenic phenotype, and that abnormalities in *APC*, *ras* and *DCC* are not necessarily sufficient for a tumorigenic phenotype.

Targeting of the *k-ras* gene in the colon cancer cell lines DLD-1 and HCT116 (Shirasawa et al., 1993) suggests that in cells prior to correction of the *ras* mutation, the genetic background on which the mutation exists was important. Correction of either the *p53* or *DCC* gene abnormality in these lines may also have resulted in a

reduced tumorigenic phenotype. As both *APC* and *ras* gene alterations are considered early events in colorectal tumorigenesis, they may represent 'gateway' options which allow dysregulation of the other genes commonly associated with colon cancer. It is also probable that different cell lines have different requirements for malignant progression, or additionally it is possible that genes other than *APC* on chromosome 5 mediate the tumorigenic response of cell lines such as SW480 and COKFu. The experiments outlined above may help explain some of the questions raised above.

An important point to consider is why the apparently normal DNA from an individual with a sporadic colon tumour yielded a mutant APC fragment. This may be explained by technical error either due to a mix up in DNA sample within our tumour bank, or an artefact of the PCR and cloning methods. However, multiple PCR reactions were pooled prior to cloning of the fragment into the pPNT vector and at least two separate clones have been analysed for the mutation which was identified in both (additionally, recent SSCP analysis has indicated different mutations in normal and tumour tissue from this patient-data not shown). This suggests that the mutation exists in the normal cells of the individual and for this reason the patient was studied further. Mutational analysis of the tumour from this patient detected a CAG-TAG Gln-Stop mutation at codon 1338, but not the truncating mutation at codon 1359 detected in the normal sample. However, recently this patient has been diagnosed as having a family history of colorectal cancer, mandibular osteomas and congenital hypertrophy of the retinal pigment epithelium (CHRPE) consistent with extracolonic

manifestations seen in Gardner's Syndrome although with no associated polyposis. Such patients have been found to be carriers of defective DNA repair genes more frequently than the normal population, and are at risk of being mosaics. This patient could therefore potentially be an *APC* mutant mosaic with tissues of different lineages containing different *APC* mutations, a situation previously described anecdotally although the frequency of tissue mosaicism is unknown (Mandl et al., 1994).

Although incomplete, the experiments described in this section emphasise the following points:

- 1.) The value of a targeting approach in the analysis of *APC* in colorectal neoplasia, as well as the potential difficulties of selecting cells with correctively targeted suppressor loci.
- 2.) The need for the generation of vectors to control for this by the replacement of one *APC* mutation with another within the same region of the gene.
- 3.) The necessity for confirmed wild type sequence correcting vectors.
- 4.) The unexpected possibility of the discovery of a patient exhibiting *APC* mosaicism.
- 5.) Accurate characterisation of a commonly used colorectal cancer cell line with respect to its *APC* status.

**In summary, the generation of two hit and run gene targeting vectors will allow functional analysis of the role of both mutant and wild type APC protein in a colon carcinoma cell line. Questions may then be asked regarding the role of**



APC mutations as an early and possibly initiating event in colorectal tumorigenesis.

## Chapter 6

### General Conclusions

This thesis contains separate studies on three tumour suppressor genes previously implicated in colorectal tumorigenesis.

Firstly, the relationship between mutation of the *p53* gene and stabilisation of p53 protein was addressed in a series of colorectal carcinomas. The general assumption that a stabilised p53 product was always equivalent to a mutated gene was shown to be false. Although there is a high correlation between mutation of the gene and stabilisation of p53 in colorectal tumours, mutation and stabilisation occur independently in a minority of cases. Specific circumstances exist in which mutated p53 protein is not stabilised, notably with exon 6 mutations. Similar previous studies on colorectal and ovarian neoplasms also commonly revealed mutations of *p53* in exon 6 with no stabilisation of the protein detectable (Kikuchi-Yanoshita et al., 1992; Cunningham et al., 1992; Teneriello et al., 1993). This suggests a region of the *p53* gene not essential for the maintenance of a stabilised conformation of the protein. Exon 6 is not located in one of the five conserved regions of the gene, perhaps suggesting a less functionally important region of the gene compared to exons 5, 7 and 8 (Soussi et al., 1990). This could be reflected in the protein structure and conformation. The recent publication of the 3-dimensional structure of the p53 protein

lends support to this idea and the mutations detected in exon 6 in this study would be located in regions not directly involved in DNA binding (Cho et al., 1994). Conversely, tumours were shown to exist with stabilised protein product but with no apparent mutation. It is possible that mutations existed outwith the region of the gene studied. For example, 5' and 3' untranslated regions, and the coding regions outwith exons 4-9 not examined in all cases. This criticism cannot be resolved by this study. It is possible, however, that non-mutational methods of stabilisation occur. This has been demonstrated by the binding of both viral and cellular oncoproteins such as the SV40 T antigen and the MDM-2 oncogene product (Sarnow et al., 1982; Momand et al., 1992). It is probable that additional cellular oncoproteins that bind p53 exist. Alternatively, errors in protein degradation could result in an accumulation of non-mutated p53 protein.

Recently the effect of the cellular environment on stabilisation of the p53 protein has been considered. It has been proposed that it is not mutation of the gene that creates a stabilised product but the environment of the cell. The mutation itself results in an inability to correctly respond to the stabilisation stimulus (Vojtešek and Lane, 1993). It may be extrapolated that the inability of the cell to respond to the stabilising stimulus due to a lack of functional p53 may itself create a permanent stimulus to the cell to upregulate and hence stabilise p53 protein.

The effect of stabilised p53 protein, either wild type or mutant, should therefore be considered in the context of the cell in which it occurs. p53 is stabilised physiologically in a number of circumstances (for example, in skin keratinocytes after UV irradiation), but the stabilisation is transient and levels return to normal after 360 hours (Hall et al., 1993). In contrast, the majority of colorectal cancer cells have permanently stabilised p53 protein. Additionally, many tumour cells exhibit genomic instability in the form of aneuploidy and gene amplifications, and p53 has often been proposed to be involved in maintaining genome integrity (Carder et al., 1994; Livingstone et al., 1992). However, it is unclear whether loss of p53 function creates genome instability, or whether the loss of p53 is a requirement for the viability of unstable cells that would, under normal circumstances die. These represent subtly different roles for the protein. The first suggests p53 defects are a causative agent of genome instability (perhaps through permitting imprecise repair or inappropriate recombination events), whilst the second proposes a role for p53 as a monitor of genome integrity coupled to the activation of apoptosis. Experimentally they are difficult to distinguish. Whether cause or effect, stabilisation of p53 protein is a physiological response to a stimulus that probably involves DNA damage, and therefore studies on stabilisation of p53 protein should be interpreted after consideration of the cellular environment as well as the mutational status of the *p53* gene.

Secondly, the role of *MCC* was studied in a small subset of colorectal tumours from non-polyposis patients who presented with colorectal cancer at a young age (<48 years). Only one of these patients fulfilled all the Amsterdam criteria for HNPCC (Vasen et al., 1991) although one other patient had an affected first degree relative. No mutations of the gene were found in the entire coding region, although a novel polymorphism in intron 13 was identified. The method of analysis used in this study has previously identified greater than 70% of point mutations present in the *p53* gene in a series of colorectal cancers. Therefore, if *MCC* was inactivated by mutation in a significant percentage of cases then this technique of PCR-SSCP would have been expected to detect the abnormality. Previous studies from other groups have failed to identify mutations of the gene in any FPC patients and the number of somatic mutations of *MCC* identified in sporadic colorectal tumours is low. A previous study from this laboratory showed that losses of 5q21 invariably involved both the *MCC* and *APC* genes (Curtis et al., 1994). It remains possible that *MCC* plays a role in a minority of colorectal tumours because firstly, deletions of 5q21 often involve both *APC* and *MCC* and secondly, mutations of the gene have been shown in a small number of carcinomas (Kinzler et al., 1991b). LOH studies, although often informative for the potential location of tumour suppressor genes may also reflect regions of the genome more susceptible to breakage than other areas and this could explain the common finding of LOH at 5q21 which includes the *MCC* gene. The small numbers of *MCC* mutations discovered to date may also be explained in light of the recent discovery that as many as 20% of sporadic colorectal tumours may be caused

by errors in mismatch repair genes (Lothe et al., 1993) and the tumours reported with *MCC* mutations may simply represent a more widespread instability generated by errors in mismatch repair or processes. The results from both this and previous studies suggest that *MCC* is not an important tumour suppressor gene in colorectal tumours.

Finally, experiments were designed to address the question of a possible role for *APC* mutation in a colon carcinoma cell line. Two targeting vectors have been created which may be transfected into the SW480 cell line in a 'hit and run' homologous recombination experiment to assess the role of APC in colorectal neoplasia *in vitro*. The first vector was designed to correct a known truncating mutation within exon 15 of the *APC* gene at codon 1338 and was inadvertently found to contain a novel truncating mutation, 21 codons downstream of this site at codon 1359.

This, however, provided a useful control as it is more difficult to design experiments that aim to correct genes with suppressor activity than it is to destroy their function. This is especially true when the suppressor gene is associated with a decrease in cell proliferation or an increase in apoptosis, both of which characteristics would render the cell disadvantaged *in vitro* when compared to cells retaining a mutant protein. The generation of a targeting vector that could assess the efficiency with which homologous recombination occurs without placing the cell at a potential growth disadvantage provides a good control for the experiment. This vector could also be used to generate *APC* mutations in otherwise 'normal' diploid cell lines to mimic the early stages of colorectal tumour development seen in both FPC and sporadic tumours. The mutation in *APC* in pPNT.APC occurs in the MCR region of the gene, a region in which greater than 60% of somatic APC mutations have been shown to occur in sporadic colorectal cancer (Miyoshi et al., 1992b). These cells would

therefore represent an event frequently seen early in sporadic colorectal cancer development, and could provide valuable information on the early progression of colorectal cancer with the subsequent accumulation of additional genetic events. A potential source of such cells may be from the "*Immortomouse*". This mouse has a SV40 T antigen transgene incorporated under the MHC promoter which is temperature and interferon  $\gamma$  sensitive (Jat et al., 1991). Fibroblast lines from the mouse could be maintained *in vitro* by culture at the permissive temperature and in the presence of interferon  $\gamma$ . Transfection of pPNT.APC and subsequent down-regulation of the SV40 T antigen would allow the effects of a mutant *APC* gene to be considered. Additionally, cells containing both wild type and mutant APC protein will provide data on the possibility of dominant negative mutation of *APC*. Removal of the second copy of *APC* by for example a deletion vector may mimic the situation seen in adenomas that have lost both copies of the *APC* gene. This will provide *in vitro* information on the question of whether inactivation of both copies of the *APC* gene is necessary for adenoma formation. Transfection with similar vectors disrupting other genes commonly mutated in colorectal cancer such as *ras* and *p53* would provide additional information on the progression of colorectal tumours. Alternatively, transfection of the mutant *APC* vector on to other genetic backgrounds such as fibroblasts from Li-Fraumeni patients or *ras* transfected cells may provide data on the way these genetic lesions functionally interact. This could then be compared to the theory that it is the total number of genetic lesions acquired in colorectal tumorigenesis rather than their order which is important (Vogelstein et al., 1988). If this theory is correct, one question that may be asked is why there is a preferred order of genetic lesions in colorectal cancer development rather than a random accumulation and it is possible that the order of accumulation of genetic events produces characteristic phenotypes

A second targeting vector containing putative wild type *APC* sequence has been generated and will be used to correct the *APC* gene in SW480 colorectal tumour cells. This will also provide evidence for the progression of colorectal cancer. Correction of a genetic lesion frequently associated with the early stages of colorectal tumorigenesis, in a cell line with multiple genetic abnormalities should provide data on the functional interaction of the associated gene products in neoplasia. The question then to be asked is whether mutation or loss of *APC* creates a 'gateway' through which subsequent genetic lesions like mutation of *K-ras* or loss/mutation of *p53* can arise. The fact that mutation of *APC* is so commonly the first genetic abnormality associated with colorectal neoplasia in both sporadic or hereditary forms suggests a critical role for the protein in tumour progression. The generation of the two targeting vectors described may help to provide further information on these questions.



## **Chapter 7**

### **Materials and Methods**

#### **7.1 The relationship between *p53* mutation and protein stabilisation in colorectal carcinomas.**

##### **7.11 Immunocytochemistry**

Immunocytochemistry had previously been performed on periodate lysine paraformaldehyde dichromate (PLPD) fixed, paraffin embedded tumour sections with the monoclonal antibody PAb1801 (Oncogene Science) as previously described (Purdie et al., 1991; Carder et al., 1993). Briefly, PAb1801 was applied to the tissue sections and a biotinylated rabbit antimouse secondary antibody linked to avidin was applied, conjugated with horse radish peroxidase and visualised by diaminobenzidine. ICC with the antibodies DO-7 (DAKO) and CM-1 (D. Lane) was performed on formalin fixed tissue in the same way except DO-7 sections were pre-treated with microwaves. Sections were incubated in a standard citrate buffer (Appendix) and exposed to microwaves in 3X5 minute treatments in a 650W microwave on high power as described (Shi et al., 1991; Cattoretti et al., 1994)

**Table 4. Antibody epitopes and details:**

ANTIBODY	EPITOPE (amino acids)	DETAILS	REFERENCE
PAb1801	32-79	Murine human specific monoclonal antibody that recognises both mutant and wild type protein.	Banks et al., 1986
D0-7	1-45, probably 37-45	Murine human specific monoclonal antibody that recognises both mutant and wild type protein.	Vojtešek et al., 1992
CM-1	pan specific	Rabbit human specific polyclonal antibody that recognises both mutant and wild type protein	Midgley et al., 1992

## 7.12 PCR-SSCP

Genomic DNA was extracted using a proteinase K digestion method. Briefly, normal mucosal or tumour tissue was digested overnight at 55°C in lysis buffer (Appendix) containing 200µg/ml proteinase K. After 16 hours an equal volume of phenol was added, mixed and centrifuged at 13k rpm for 5 minutes. The upper phase was removed and an equal volume of phenol:chloroform (1:1) added mixed and centrifuged as above. The upper phase was again removed and an equal volume of chloroform:isoamylalcohol (24:1) added and treated as above. An equal volume of isopropanol was added to the upper phase and mixed until a white precipitate formed. The sample was centrifuged as above and the supernatant removed. The pellet was

washed in 70% and then 100% ethanol and allowed to dry. The DNA pellet was resuspended in TE buffer (Appendix).

The polymerase chain reaction was performed on 0.1-1µg paired normal and tumour genomic DNA samples in an 100µl reaction containing 20nmoles of each deoxynucleotide (Pharmacia), 50pmoles of each primer (Oswel DNA services), and 2 units of a thermostable Taq polymerase in the relevant buffer (Life Technologies). The samples were overlaid with paraffin oil to eliminate sample evaporation. PCR was performed in a DNA thermocycler (Hybaid) with the following temperature profile: 1 cycle of 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute and 1 cycle of 72°C for 10 minutes. The primers were as follows (5' to 3'):

exon 5up TTCCTCTTCCTACAGTAGTC

exon 5reverse CGATGGTGAGCAGCTGGG

exon 6up CCTCACTGATTGCTCTTAGG

exon 6reverse CTGAGGTCTGGTTTGCAACT

exon 7up TGTGTTATCTCCTAGGTTGG

exon 7reverse GTCAGGAGCCACTTGCCA

exon 8up TCCTATCCTGAGTAGTGGT

exon 8reverse CGAGGTAAGCAAGCAGGA.

The samples were extracted once with 24:1 chloroform/isoamylalcohol to remove mineral oil. 5µl-10µl were then denatured in 80µM NaOH, 10µM EDTA at 48°C for 5 minutes. 2µl sequencing stop solution (Appendix) was added, and the whole sample

loaded onto a 5% glycerol, 1xTBE (NBL), 0.5X MDE *Hydrolink* gel (Hoefer). The gel was run in 1xTBE on the SE600 PAGE apparatus (Hoefer) at 25°C, 20W for 2-3 hours. (additionally, alternative conditions of 4°C in the absence of glycerol were used in some cases). Bands were visualised by a silver stain (BioRad) as per manufacturers instructions. Briefly, the gel was fixed in a 40% methanol solution on an orbital shaker for at least 1 hour or overnight, followed by two subsequent fixes in a 10% ethanol solution for 30 minutes. The gel was then oxidised in a solution containing potassium dichromate and nitric acid for 10 minutes. 12-15 two minute washes of deionised water were then applied to the gel until all the yellow colour was removed. A silver nitrate solution was then applied for 30 minutes and removed by a 2 minute deionised water wash. Developing solution containing sodium carbonate and paraformaldehyde was added in 2 batches and left until the brown DNA bands were visible but before a high level of background was observed. The reaction was stopped using a 5% acetic acid solution for 30 minutes. The gel was dried onto Whatmann 3MM paper and laminated.

### 7.13 Sequencing of tumours with abnormal SSCP bands

Sequencing was performed by one of two methods using the PCR primers as sequencing primers:

#### 1.) Subcloning of a PCR product containing exons 7-9 of the p53 gene into pGEM7Zf+

A PCR product containing exons 7-9 of the p53 gene was cloned into the pGEM-Zf7+ plasmid vector (Promega) after amplification using the above PCR conditions and the following primers

890- 5'GAGAGAAGCTTGCCACAGGTCTCCCCAAGGCGAA

891- 5'AGAGAGGGATCCACTTTCCACTTGATAAGAGGTCCCAAG

The PCR product was blunt ended using 0.5mM dNTP and 5U of Klenow fragment (Life Technologies) in the EcoRI restriction enzyme buffer (Boehringer Mannheim) at 30°C for 15 minutes. The reaction was stopped by heating at 75°C for 10 minutes. The resulting blunt ended PCR fragment was cloned into the SmaI restriction enzyme site of the pGEM7Zf+ vector by a blunt ended ligation using 5U of T4 DNA ligase in the appropriate buffer (Boehringer Mannheim). Ligations were diluted 1/5 and used to transform DH5 $\alpha$  competent cells as per the manufacturers protocol (Life Technologies). Briefly, 1-10ng of diluted ligated DNA was added to 20  $\mu$ l of DH5 $\alpha$  cells in a chilled microfuge tube and left on ice for 30 minutes. Cells were heat shocked at 42°C for 40 seconds and returned to ice. 80 $\mu$ l of SOC medium (Appendix) was added and the tubes were placed in a 37°C orbital shaker for 1 hour.

The cells were applied to L-Amp plates (Appendix) with 4 $\mu$ l of 200mg/ml IPTG (isopropylthio- $\beta$ -D-galactoside) and 40 $\mu$ l of 20mg/ml X-Gal (5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside) for blue white colony screening (Promega) and left at 37°C overnight. Briefly, cloning into the polylinker of pGEM7Zf+ disrupts the vector *lac $\alpha$*  gene. This leads to a loss of functional  $\alpha$ -peptide with no resulting complementation with the host cell *lacZ* gene product to produce functional  $\beta$ -galactosidase when plated with X-Gal and IPTG. Resulting colonies are therefore white not blue. Plates were placed at 4°C for 8 hours and approximately 100 white colonies were pooled and used to seed 10ml L-broth (Appendix) with 10 $\mu$ g/ml ampicillin for selection of plasmids which contains the bacterial ampicillin resistance gene.

Plasmid DNA was prepared using the Wizard Mini-prep kit (Promega). Briefly, 1-3mls of cells were pelleted and resuspended in 200 $\mu$ l Cell Resuspension solution (Appendix). The cells were lysed by the addition of 200 $\mu$ l Cell Lysis buffer (Appendix) and mixed by inverting gently 4-5 times. The samples were neutralised by the addition of 200 $\mu$ l Neutralisation solution (Appendix). Precipitated proteins were pelleted by centrifugation and the supernatant added to 1ml of Wizard Mini-prep resin. The mix was then applied to the Mini-prep column and applied to the column using a 2ml luer syringe. 2 mls Column Wash solution (Appendix) was applied to the column. Residual Column Wash was removed from the resin by centrifugation. Plasmid DNA was eluted by the addition of TE buffer heated to 65°C, left for 1 minute, centrifuged and collected.

DNA was sequenced by the chain termination method using the PCR-SSCP primers as sequencing primers and ( $^{35}\text{S}$ )dATP (Amersham) as per the manufacturers protocol for dsDNA (United States Biochemicals, Sequenase Version 2.0). Briefly, 3-5 $\mu\text{g}$  DNA were added to 1X Reaction Buffer (Appendix), 1pmole primer and deionised water to a volume of 10 $\mu\text{l}$ . Primer was annealed to the DNA by heating to 60°C and cooled to less than 35°C over 30 minutes. Samples were then placed on ice and 0.1M dithiothreitol, 1X Labelling Mix (Appendix), 5 $\mu\text{Ci}$  ( $^{35}\text{S}$ )dATP and Sequenase enzyme added and mixed gently. Samples were labelled at room temperature for 2-5 minutes. Reactions were terminated by the addition of 3.5 $\mu\text{l}$  labelled mix to 2.5 $\mu\text{l}$  of each dideoxynucleotide at 37°C for 5 minutes. The termination reaction was stopped by the addition of 4 $\mu\text{l}$  of sequencing stop solution. Samples were maintained at -20°C until required. Samples were denatured at 95°C for 5 minutes, and separated by electrophoresis through a prewarmed 50°C 6% urea/acrylamide gel (Appendix). After electrophoresis, the gel was fixed for 10 minutes in a 10%methanol/10%acetic acid solution, dried onto Whatmann 3MM paper, and exposed to autoradiograph film overnight.

## **2.) PCR amplification of exons 5-6 using a biotinylated upstream primer and separation of the DNA strands using Dynabeads.**

Exons 5-6 of p53 were amplified using a biotinylated upstream primer as follows

5'AGGAGGTGCTTACACATGTT

with the exon 6 reverse primer as above under the above PCR conditions. The DNA was separated into single strands by the use of magnetic Dynabeads as per the manufacturers instructions (Dyna). Briefly, the biotinylated PCR products were mixed with 25 $\mu$ l of avidin coated magnetic beads previously washed in 100 $\mu$ l TES (Appendix). The biotinylated DNA was allowed to absorb onto the avidin coated beads for 5 minutes at room temperature. The supernatant was removed using a magnet to 'fix' the DNA attached beads to the side of the tube. The DNA strands were separated by the addition of freshly prepared 0.15M NaOH at room temperature for 5 minutes. The supernatant containing the complementary single stranded DNA was recovered using a magnet and ethanol precipitated with 200 $\mu$ l 100% ethanol at -20°C for 2 hours. The complementary DNA was washed with 70% ethanol and resuspended in 20 $\mu$ l distilled water. The biotinylated DNA strand remained bound to the avidin coated beads and was washed with 100 $\mu$ l TES followed by 100 $\mu$ l of distilled water and resuspended in 20 $\mu$ l water. Sequencing was performed as above.



## **7.2 Is the *MCC* gene involved in Colorectal Carcinogenesis?**

### **7.21 Clinical material and DNA preparation**

The xenografts were passaged in SCID mice by techniques previously described (McQueen et al., 1991). Patients were selected on the basis of their age at diagnosis. Family histories were established by interview of the patients and relatives and confirmed by reference to histopathology reports and death registers (Data collected by Norma Brown).

High molecular weight DNA was prepared from xenograft tissue as previously described. DNA was extracted from corresponding blood samples by the addition of blood lysis buffer (Appendix) for 30 minutes. An equal volume of TE saturated phenol was added and mixed thoroughly then centrifuged at 3k rpm for 20 minutes. The upper layer was removed and to it was added 0.5X 7.5M ammonium acetate and 3X cold isopropanol. This was mixed and left at -20°C overnight. The DNA pellet was recovered by centrifugation as above, dried and resuspended in TE.

### **7.22 PCR-SSCP of exons 1-17**

The polymerase chain reaction was performed on 0.1-1µg paired blood and xenograft genomic DNA samples, in a 50µl reaction containing 20nmoles of each deoxynucleotide, 50pmoles of each primer, and 2 units of a thermostable Taq polymerase in the relevant buffer. PCR was performed in a DNA thermocycler with the following temperature profile: 1 cycle of 94°C for 5 minutes, 30 cycles of 94°C

for 1 minute, an annealing temperature calculated by subtracting 2°C from the melting temperature (see below) of each primer for 1 minute, 72°C for 1 minute and 1 cycle of 72°C for 10 minutes. The primers were as indicated (Table 5). The melting temperature for a given primer can be roughly estimated using the following formula: Melting temperature ( $T_m$ ) in °C = 4(total number of G and C residues) + 2(total number of A and T residues).

The samples were prepared for SSCP analysis as previously described. SSCP gels were run in 1xTBE on the SE600 PAGE apparatus (Hoefer Scientific) at 25°C, 20W for 2-3 hours. Additionally alternative conditions of 4°C in the absence of glycerol were used in some cases. Bands were visualised by a silver stain as previously described. The gel was dried onto 3MM paper and laminated.

### **7.23 Sequencing of polymorphic exons**

Sequencing was performed as previously described either by Dynabead separation (Dyna) or by subcloning of the PCR fragment in to a plasmid vector (pGEM-7Zf(+), Promega). Samples were then sequenced by the chain termination method using Sequenase Version 2.0 as above (United States Biochemicals).

**Table 5. PCR PRIMERS AND SIZE OF AMPLIFICATION  
PRODUCT FOR MCC EXONS.**

EXON	SIZE IN BASE PAIRS	SEQUENCE (5' to 3')
1 UP 1 REVERSE	174	TGGCAGAAGGGACAAG GAAACCCTAGCTCCCGAC
2 UP 2 REVERSE	198	GGAAGGAAATACATCTCTG GCAAACATAAAAACCTTTCCAG
3 UP 3 REVERSE	243	GAATTCATCAGCACTTCT TCAGCTCCAAGATGGAGGG
4 UP 4 REVERSE	234	CTCTAGGGGGTTGTCTTAT ACTCTGGCTTGGTGGCAA
5 UP 5 REVERSE	237	CAGCATTTGTTGCCCTGTG CCTCACAGAGGGTGAGG
6 UP 6 REVERSE	264	TTTTCTTTGGGGCTGTGTTTT CAGGAAGGGCCATCCCCA
7 UP 7 REVERSE	221	GCCAGGGAAACCACCAAC TCATCGGGTCCATCCCCA
8 UP 8 REVERSE	186	CTGAGGAGTGGTGTGTAGT TGTAGCTCTGCCGGGTAC
9 UP 9 REVERSE	221	GTTCCAAAGGGGAATTAATG CTCCCTGCCCCACAATCA
10 UP 10 REVERSE	199	GTAACCCTAGTGTGAATCTGA GCCTCGCGCTGTCTTCC
11 UP 11 REVERSE	146	CCCATGTGCTTTGT AGAGGGACTCTGGAGACA
12 UP 12 REVERSE	245	ATGTTGATTAATCCGTTGGC ACCCAGAGCAGAAGGCT
13 UP 13 REVERSE	307	GAATAACACAGGGTGGTGG CCTAGCACATGGGACCCAC
14 UP 14 REVERSE	289	GCCTGCTCTCAGATCGGGCA AAGGGTGTCCCCAAGCCA
15 UP 15 REVERSE	173	GGCCTAACTGGAATGTGT GCCAGATAAACACCAGC
16 UP 16 REVERSE	205	GGGCACCTCCAGCATCAT CGGTGGGGGCTGGGTATA
17 UP 17 REVERSE	233	GAATCTTAATACCAATTTCCC CGGTGCGTGAGTGCTGAT

## **7.3 Targeting of the *APC* gene in a colon carcinoma cell line**

### **7.31 Generation of a hit and run targeting vector pPNT.APC**

The vector pPNT.APC was generated by insertion into the plasmid pPNT of a 2.8kb PCR product amplified from genomic DNA from exon 15 of the *APC* gene nucleotides 3232-6017. This region spans the truncating mutation at codon 1338 of *APC* in SW480 cell line. pPNT is a pUC based vector containing the neomycin resistance gene *neo* and the Herpes Simplex Virus thymidine kinase gene *tk*, both under a PGK promoter (obtained from the Centre for Genome Research Edinburgh, S. Fiering).

PCR amplification of the region of *APC* exon 15 (nucleotides 3232 to 6017) was performed using 1µg of 'normal' mucosal genomic DNA prepared as above in a 50µl reaction containing 200µM of each deoxynucleotide, 50pmoles of each primer, and 2 units of a thermostable Taq polymerase in the relevant buffer. PCR was performed in a DNA thermocycler with the following temperature profile: 1 cycle of 94°C for 5 minutes, 4 cycles of 94°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, 26 cycles of 94°C for 1 minute, 48°C for 1 minute, 72°C for 1 minute, and 1 cycle of 72°C for 10 minutes.

The primers were as follows (5' to 3'):

SW480APCup.5 TATGAATTCTATACTGAGAGCACTGATG

SW480APCrev.2 ATATGGATCCTGATGCTTGAGGTTT

The primers were designed to include restriction enzyme sites (bold type) at either end to allow directional cloning of the fragment into the plasmid vector (SW480APCup.5 contains a BamHI site whilst SW480APCrev.2 contains an EcoRI site). The 2.8kb PCR fragment was extracted once with 24:1 chloroform:isoamyl alcohol to remove any mineral oil from the PCR reaction. The fragment was then separated from other PCR reaction constituents by cleaning with a Wizard Clean-Up column as per the manufacturers protocol (Promega). Briefly the sample is mixed with the Wizard Clean-up resin and applied to the column as described above for the Wizard Mini-prep procedure. The column is washed with 2ml 80% isopropanol and the sample collected as described. Both the eluted PCR fragment and 5µg of pPNT were digested with the appropriate amount of the restriction enzymes BamHI and EcoRI in BamHI buffer (Boeringer Mannheim) at 37°C for four hours.

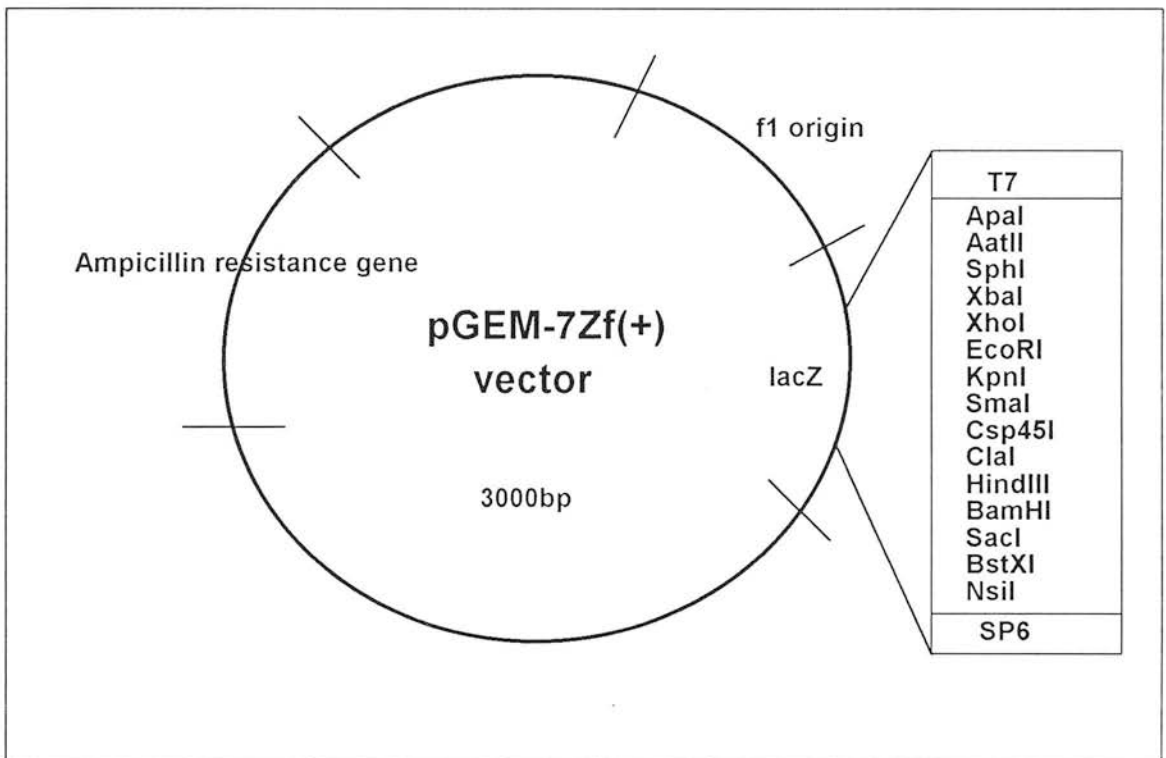
The digestion reaction components were separated from the PCR fragment and the vector using the Wizard Clean-up columns as described above and quantified against known DNA standards. Samples containing 1:1, 3:1 and 10:1 molar ratios of PCR fragment to vector and 5-10U T4 DNA ligase in an appropriate buffer were placed at 16°C overnight. 1/5 dilutions of the ligation mixes were used to transform DH5α competent cells as previously described. Plasmid DNA was extracted using the Wizard Miniprep Kit. Eluted plasmid DNA was digested with both BamHI or EcoRI or both as described above, mixed with 2µl loading buffer (Appendix) and separated by electrophoresis through a 0.8% agarose gel (Flowgen) to identify plasmids

containing the 2.8kb APC fragment. Plasmids containing the fragment were replated and regrown.

Maxipreps of pPNT.APC were prepared according to the manufacturers protocol (Qiagen Tip 500). Briefly, 500ml of cells were pelleted by centrifugation at 4°C for 15 minutes at 6000xg. The supernatant was removed and the cells resuspended in 50ml buffer P1(Appendix). The cells were lysed by the addition of 50ml of buffer P2 (Appendix), mixed gently and left at room temperature for 5 minutes. The sample was neutralised by the addition of 50ml of chilled buffer P3 (Appendix), mixed and left on ice for 30 minutes. The precipitated proteins were pelleted by centrifugation at 4°C for 2X 15 minutes at 20,000xg. A QIAGEN-tip 2500 was equilibrated by the addition of 35ml of buffer QBT (Appendix) and the column allowed to empty by gravity flow. The clear supernatant was applied to the column and again allowed to empty by gravity flow. The sample was washed by 4X 50ml buffer QC (Appendix) and the sample finally eluted with 35ml buffer QF (Appendix). The DNA was precipitated with 0.7 volumes of isopropanol centrifuged at greater than 20,000xg at 4°C for 30 minutes. The DNA pellet was washed with 5ml cold 70% ethanol and air dried before resuspension in TE buffer.

The 2.8kb APC fragment in pPNT.APC was sequenced using the Erase-a-Base system (Promega). This involves the generation of plasmids containing unidirectional nested deletions of the insert DNA using exonuclease III (Exo III). ExoIII digestion proceeds at a uniform rate at a given temperature and removal of timed aliquots allows deletions of a predetermined length to be created. An adjacent sequencing

primer site is protected from digestion by creating a 4-base 3' overhang by restriction digestion. The ends of the deleted sequences are repaired and the plasmid used to transform competent cells as above. The 2.8kb APC fragment was excised from pPNT.APC by a BamHI/EcoRI double digest as previously described and cloned into the vector pGEM-Zf7+. The pGEM7Zf+ vector contains a number of unique restriction enzyme sites suitable for sequencing by this method.



**Figure 25.** A map of the vector pGEM7Zf(+) as shown in Erase-a-Base technical manual.

Nicked and linear DNA was removed from the supercoiled plasmid DNA by the following protocol; 0.1 volumes of 3M sodium acetate pH 5.2 were added to the plasmid DNA sample and precipitated with ethanol as described. The DNA pellet was

resuspended in deionised water and 2M sodium acetate pH 4.0 was added to a final concentration of 50mM and 2M NaCl to a final concentration of 75mM. At 4°C, an equal volume of acid-phenol (Appendix) was added and mixed. After centrifugation at 10,000xg for 5 minutes the aqueous phase was recovered and the acid-phenol step repeated 2-3 times. After extraction, 0.05 volumes of 1M Tris.HCl pH 8.6 was added and the sample extracted with 1 volume of chloroform:isoamylalcohol (24:1). To the aqueous phase, 0.1 volumes 2M NaCl was added and the DNA precipitated with ethanol. The pellet was rinsed with 70% ethanol, dried and resuspended at 0.5-1µg/µl in TE buffer.

In order to generate an exonuclease III sensitive 5' overhang, 5µg of the recovered DNA were digested with BamHI as described, overnight at 37°C. The digested DNA was extracted with phenol:chloroform (1:1) and chloroform:isoamylalcohol (24:1) and precipitated with 3M sodium acetate and ethanol. The pellet was resuspended and digested overnight with SacI to generate an exonuclease III resistant 3' overhang (see Figure 25). The digest was extracted as above and the dried pellet resuspended in 60µl exonuclease III (ExoIII) buffer (Appendix). The DNA was warmed to 37°C in a water bath and 300U ExoIII were added. At 37°C digestion proceeds at approximately 450 bases/minute with a 20-30 second lag before digestion begins. 2.5 µl samples were removed at 30 second intervals and mixed with 7.5µl S1 nuclease mix (Appendix) on ice. The samples were placed at room temperature for 30 minutes before the addition of 1µl of S1 stop buffer (Appendix) and heat inactivation at 70°C for 10 minutes. The samples were transferred to 37°C and 1µl Klenow mix added



(Appendix). After incubation for 3 minutes, 1µl dNTP mix (Appendix) was added and the samples incubated for a further 5 minutes. At room temperature, 40µl ligation mix (Appendix) was added, mixed and incubated for 1 hour. 10µl of each ligation reaction was added to 20µl DH5α competent cells and transformation proceeded as previously described. 6 transformants per time point were selected and individual colonies were picked and smeared near the bottom of a sterile microfuge tube, the remaining cells were streaked onto a fresh L-Amp plate and incubated at 37°C. 50µl 10mM EDTA pH 8.0 was added to the cells in the microfuge tube and the cells resuspended. 50µl cracking buffer (Appendix) was added and the cells vortexed. To this was added 1.5µl 4M KCl and 0.5µl of 0.4% bromophenol blue and the samples placed on ice for 5 minutes. The samples were centrifuged and 25µl of the supernatant were separated by electrophoresis on a 0.7% agarose gel. The plasmid sizes were estimated by running against known supercoiled plasmid markers (Life Technologies). Correctly sized plasmids were grown as overnight cultures and plasmid DNA was extracted as above. Sequencing was performed using 1pmole of a commercial sequencing primer (SP6, Promega) as previously described.

### **7.32 Generation of the targeting vector pPNT.APCwt**

pPNT.APCwt was generated as described for pPNT.APC above, except that the template DNA used to amplify the 2.8kb APC fragment was mucosal DNA from non-colon cancer bearing individuals (provided by M. Dunlop). pPNT.APCwt remains to be sequenced to confirm the absence of mutations.

### **7.33 Culture and analysis of SW480 cells**

SW480 cells were obtained from the Laboratories of the ICRF (C Marshall) and grown in L-15 medium supplemented with heat inactivated 10% new-born calf serum (Sigma and Life Technologies). Cultures were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub>.

Cells grew as a monolayer in isolated clumps of epithelial like cells. Tumorigenicity was assessed by the subcutaneous injection of 10<sup>7</sup> SW480 cells in 0.2ml phosphate buffered saline (PBS) into SCID mice maintained in isolators. Tumours were detected in all animals by three weeks after injection. The tumours were removed, fixed and examined histologically by A.H.Wyllie. The histological characteristics of the original parental tumour i.e. adenocarcinoma were retained (Figure 23).

#### **7.331 APC analysis of SW480**

A 2Kb APC probe was generated by PCR amplification of normal genomic DNA under the following conditions. In a 50µl reaction, 15nmoles deoxynucleotides, 50pmoles primer, and 2 units of a thermostable Taq polymerase in the relevant buffer

were amplified in a DNA thermocycler with the following temperature profile: 1 cycle of 94°C for 5 minutes, 4 cycles of 94°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, 26 cycles of 94°C for 1 minute, 48°C for 1 minute, 72°C for 1 minute, and 1 cycle of 72°C for 10 minutes. Primer sequences were as follows (5'-3').

630N CACCGAATTCGAATACTACAGTGTTACC

631N AAGAGGATCCATCTGGAGTACTTTC

Amplified product was cloned into pGEM7Zf+ as previously described to generate a vector pAPC.KC. Plasmid DNA was extracted as described and labelled with <sup>32</sup>P by nick translation using the Megaprime kit as per manufacturers instruction (Amersham). Briefly, 25ng of plasmid DNA were denatured at 100°C for 5 minutes in the presence of random nonamer primers in a 50µl reaction volume. The DNA and primers were allowed to anneal at room temperature and 10µl Megaprime reaction buffer (Appendix) added. 50uCi (<sup>32</sup>P) dCTP and 2U Klenow fragment were added, mixed and placed at 37°C for 30 minutes.

SW480 DNA was prepared by the proteinase K method previously described. DNA was digested with PstI in an appropriate buffer (Boehringer Mannheim) and the digests electrophoresed with 1/5 volume loading buffer on a 0.8% agarose gel. The gel was depurinated in 0.25M HCl for 10 minutes and rinsed in distilled water. A capillary blot was set up for an alkali transfer using 0.4M NaOH and the DNA transferred to a Hybond N+ (Hybond) membrane by capillary action overnight. The membrane was briefly rinsed in 2XSSC (Appendix) and dried between sheets of 3MM paper. Radiolabelled probe was denatured at 100°C for 5 minutes prior to addition to

the filter after prehybridisation of the filter in hybridisation solution (Appendix) at 65°C in hybridisation bottles (Hybaid). The probe was hybridised to the DNA on the filters at 65°C overnight. The next day, the probe was removed and the filter washed in 2xSSC at 65°C for 30 minutes and then 2xSSC, 0.5% SDS at 65°C for 2 hours (constituting a medium stringency wash). Filters were wrapped in Saran wrap and placed in a light proof cassette with X-OMAT film (Kodak). X-ray film was developed after 24 hours.

### **7.332 Fluorescence in-situ Hybridisation (FISH) analysis of APC in SW480**

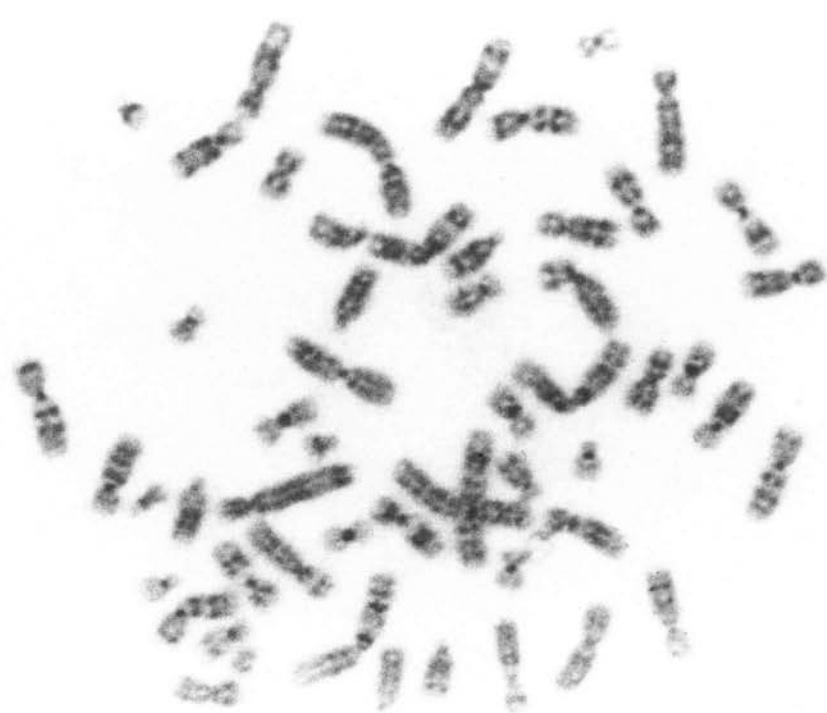
Metaphase spreads of SW480 were prepared by the addition of 1µg/ml colchicine to the culture medium. Cells were left at 37°C for 2 hours, rinsed in PBS and harvested using 0.2% trypsin. Cells were centrifuged at 2.5K for 5 minutes and swollen by the addition of 10ml fresh hypertonic 0.075M KCl for 10 minutes. Cells were centrifuged at 2.5K for 5 minutes and the KCl removed. The cell pellet was disrupted by gentle tapping of the tube and 3;1 methanol to glacial acetic acid fix was added drop by drop. Centrifugation and addition of fix was repeated 3X. Spreads were obtained by dropping the cell/fix solution from approximately 2 metres onto ice-cold, clean microscope slides and allowed to dry (Figure 26).

YAC and cosmid APC clones were used as probes for FISH analysis.

The YAC clone was obtained from K.Kinzler and isolated from yeast chromosomes by S.Farrington. Briefly this involved the growth and lysis of yeast cultures and

**Figure 26. Metaphase spread of SW480 chromosomes**

A metaphase spread of SW480 cell with 55 chromosomes.



separation under pulsed field gel electrophoresis against yeast markers. The gel was fixed and the DNA transferred to a nylon membrane. Southern analysis was performed with the 2kb APC probe to locate the APC YAC DNA (data not shown). The gel was rerun and a gel slice containing the YAC DNA (calculated from the previous gel) was extracted. Preparation of YAC DNA involved the digestion of the agarose, and subsequent addition of linker sequences to the DNA to provide PCR primer sites. A PCR reaction was then performed and the amount of DNA generated was estimated.

The APC cosmid was prepared as described in Hampton et al., 1992 and Ward et al., 1993 and was performed by J. Fantes.

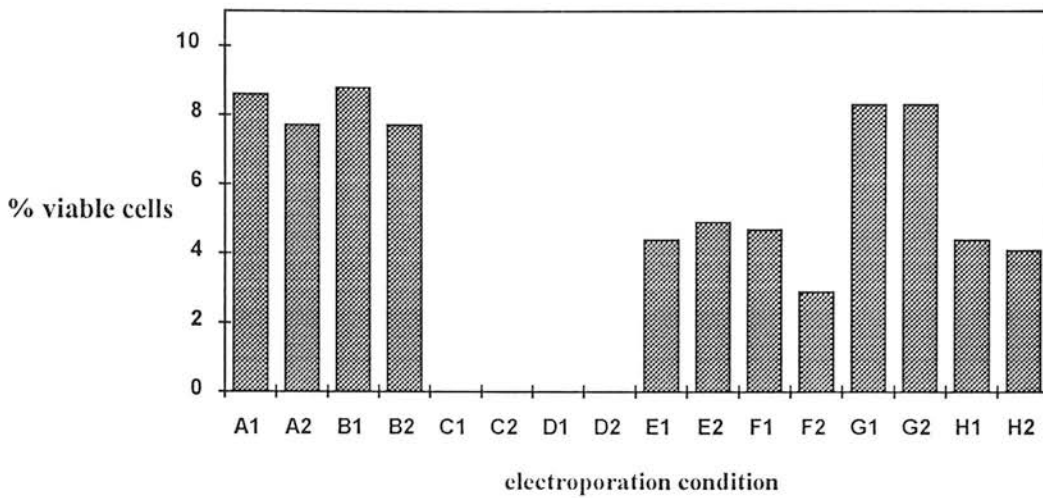
The YAC and cosmid DNA were labelled with biotin and used as probes for FISH analysis on the SW480 chromosome spreads. Briefly probes were biotinylated by nick translation, applied to the chromosome spreads, the signal amplified by avidin and biotin linked antibodies and visualised by avidin/FITC conjugated secondary antibodies. Cells were counter-stained using propidium iodide or 4', 6'-diamidino-2-phenylindole (DAPI) and observed under a Zeiss Axioplan fluorescent microscope linked to an Apple Macintosh computer with a photometric camera and Digital Scientific software or by a confocal scanning microscope (BioRad) linked to a similar computer package.

Additionally, FITC or Texas Red labelled chromosome paints were used either independently or in conjunction with the YAC or cosmid probes to identify chromosomes 5 and 20. (Cambio, as per instruction).

### 7.34 Transfection of targeting vector pPNT.APC

Electroporation conditions were established as follows. A range of conditions (voltages from 0.2-1KV and resistance from 3-500 $\mu$ F) were applied to the electroporation of 30 $\mu$ g pPNT.APC into aliquots of  $10^6$  SW480 cells. Cells were plated at densities of  $5 \times 10^5$ ,  $10^5$ ,  $10^4$ , and  $10^3$  in L-15 medium supplemented with 10% HINCS. After 24 hours, 600 $\mu$ g/ml G418 was added to the plates containing  $5 \times 10^5$  and  $10^5$  cells, and at all subsequent media feeds (every two days). After one week, the number of viable cells was calculated at all initial plating densities by washing in PBS, fixing with 70% ethanol and staining with 5% Giemsa. The number of colonies grown on the plates originally containing  $10^3$  and  $10^4$  cells were counted and the number of viable cells remaining for each electroporation condition calculated.





**Figure 27: Viability of cells at different electroporation conditions.**

Legend.

A1: 0.4KV, 3uF -  $10^4$  cells  
 A2: 0.4KV, 3uF -  $10^3$  cells  
 B1: 0.4KV, 125uF -  $10^4$  cells  
 B2: 0.4KV, 125uF -  $10^3$  cells  
 C1: 0.4KV, 250uF -  $10^4$  cells  
 C2: 0.4KV, 250uF -  $10^3$  cells  
 D1: 0.4KV, 500uF -  $10^4$  cells  
 D2: 0.4KV, 500uF -  $10^3$  cells

E1: 25uF, 0.2KV -  $10^4$  cells  
 E2: 25uF, 0.2KV -  $10^3$  cells  
 F1: 25uF, 0.4KV -  $10^4$  cells  
 F2: 25uF, 0.4KV -  $10^3$  cells  
 G1: 25uF, 0.6KV -  $10^4$  cells  
 G2: 25uF, 0.6KV -  $10^3$  cell  
 H1: 25uF, 1.0KV -  $10^4$  cells  
 H2: 25uF, 1.0KV -  $10^3$  cells

The plates containing cells originally plated at densities of  $5 \times 10^5$  and  $10^5$  and subsequently placed in G418 selection were stained as above and the number of colonies counted to obtain a rough estimate of the number of cells to be electroporated in order to obtain sufficient G418 resistant clones for analysis. Cells plated at a density of  $5 \times 10^6$  generated approximately 20 G418 resistant clones (data not shown).

pPNT.APC DNA was linearised with NsiI in an appropriate buffer for 4 hours at  $37^\circ\text{C}$  (Boeringer Mannheim).  $10^8$  SW480 cells were transfected with 800 $\mu\text{g}$  linearised pPNT.APC by electroporation at 25uF and 0.6kV in a BioRad electroporation cuvette in the Gene Pulser (BioRad). Transfected cells were plated at

cell densities of either  $10^7$  or  $5 \times 10^6$  cells per plate with culture medium. After 24 hours the medium was supplemented with  $600 \mu\text{g/ml}$  G418 sulphate (Life Technologies BRL). Medium with G418 was replaced every two days.

After three weeks colonies of G418 resistant cells were readily visible by eye and colonies were picked using a finely drawn Pasteur pipette in a still air hood and placed in 24 well plates with G418 supplemented media. Individual colonies that grew to confluence were removed by trypsinisation and half of each colony was placed in freezing media (Appendix) and frozen at  $-70^\circ\text{C}$  overnight and transferred to liquid nitrogen. DNA was extracted from the remaining cells using the proteinase K method previously described. Approximately  $5 \mu\text{g}$  of DNA was digested with *Stu*I (Boehringer Mannheim) and electrophoresed on a 0.8% gel. Alkali blotting was performed as previously described.

## **APPENDIX**

### **CITRATE BUFFER:**

7mM Citric acid pH6

### **LYSIS BUFFER:**

50mM Tris pH 8.0

50mM EDTA

100mM NaCl

5mM dithiothreitol

1% SDS

0.5 mM spermidine

### **TE:**

10mM Tris.HCl pH 7.4

1mM EDTA pH 8.0

### **SEQUENCING STOP SOLUTION:**

98% deionised formamide

10mM EDTA pH 8.0

0.025% xylene cyanol FF

0.025% bromophenol blue

### **SOC MEDIUM:**

Prepared as described in transformation protocol (Life Technologies).

2% bactotryptone

0.5% yeast extract

10mM NaCl

2.5mM KCl

10mM MgCl<sub>2</sub>

10mM MgSO<sub>4</sub>

20mM glucose

### **L-AMP PLATES:**

1% bacto-tryptone

0.5% Bacto yeast extract

1% sodium chloride

1.2% bacto agarose

10µg/ml ampicillin (added when agarose has cooled to less than 60°C).

### **L-BROTH:**

1% bacto-tryptone

0.5% Bacto yeast extract

1% sodium chloride

**WIZARD MINI-PREP SOLUTIONS****CELL RESUSPENSION BUFFER:**

50mM Tris.Hcl pH 7.5

10mM EDTA

100ug/ml RNase A

**CELL LYSIS BUFFER:**

0.2M NaOH

1% SDS

**NEUTRALISATION BUFFER:**

1.32M KAc pH 4.8

**COLUMN WASH:**

200 mM NaCl

20mM Tris.HCl pH 7.5

5mM EDTA

Dilute with 95% ethanol to a total ethanol concentration of 55%.

**SEQUENCING SOLUTIONS****5X REACTION BUFFER:**

200mM Tris.HCl pH7.5

100mM MgCl<sub>2</sub>

250mM NaCl

**5X LABELING MIX:**

7.5uM dGTP

7.5uM dCTP

7.5uM dTTP

**6% SEQUENCING GEL:**

6% acrylamide:bisacrylamide (40:1)

8M urea

1xTBE

1μl 25% ammonium persulphate per ml of gel

1μl TEMED per ml of gel

**TES:**

10mM Tris-HCl pH 8.0  
1mM EDTA  
100mM NaCl

**BLOOD LYSIS BUFFER:**

0.1M Tris pH 8.0  
20mM NaCl  
1mM EDTA  
4% SDS

**GEL LOADING BUFFER:**

30% w/v glycerol  
0.25 % xylene cyanol FF  
0.25 % bromophenol blue

**QIAGEN MAXI-PREP SOLUTIONS****P1:**

50mM Tris.HCl  
10mM EDTA pH 8.0  
100µg/ml RNase A

**P2:**

200mM NaOH  
1% SDS

**P3:**

3M KAc pH 5.5

**BUFFER QBT:**

750mM NaCl  
50mM MOPS  
15% ethanol pH 7.0  
0.15% Triton X-100

**BUFFER QC:**

1M NaCl  
50mM MOPS  
15% ethanol pH 7.0

**BUFFER QF:**

1.25M NaCl  
50mM Tris.HCl  
15% ethanol pH 8.5

**ERASE-A-BASE SOLUTIONS****ACID PHENOL:**

500ml 50mM NaAc pH 4.0 added to 500g phenol. Dissolve 3-4 hours  
 Remove upper aqueous phase, add 500ml 50mM NaAc. Repeat until pH of  
 aqueous layer is less than 4.1

**10X EXOIII BUFFER:**

660mM Tris.HCl pH 8.0  
 6.6mM MgCl<sub>2</sub>

**S1 NUCLEASE MIX:**

1X S1 buffer  
 2-3U S1 nuclease  
 deionised water to 8ul

**7.4X S1 BUFFER:**

0.3M KAc pH 4.6  
 2.5mM NaCl  
 10mM ZnSO<sub>4</sub>  
 50% glycerol

**S1 STOP BUFFER:**

0.3M Tris base  
 0.05M EDTA

**KLENOW MIX:**

1X Klenow buffer  
 3U Klenow

**KLENOW BUFFER:**

20mM Tris.HCl pH 8.0  
 100mM MgCl<sub>2</sub>

**dNTP MIX:**

0.125mM each dATP, dCTP, dGTP, dTTP

**LIGATION MIX:**

1X ligase buffer  
5% PEG  
\* 1mM DTT  
1U T4 DNA ligase  
deionised water to 40 $\mu$ l

**10X LIGATION BUFFER:**

500mM Tris.HCl pH 7.6  
100mM MgCl<sub>2</sub>  
10mM ATP

**2X CRACKING BUFFER:**

2ml 5M NaOH  
2.5ml 10% SDS  
10g sucrose  
deionised water to 50ml

**SOUTHERN HYBRIDISATION SOLUTIONS****MEGAPRIME REACTION BUFFER:**

dATP, dGTP, dTTP in concentrated reaction buffer containing Tris.HCl pH 7.5, MgCl<sub>2</sub> and 2-mercaptoethanol (as described in manufacturers protocol).

**20X SSC:**

3M NaCl  
0.3M trisodium citrate pH 7.0

**HYBRIDISATION BUFFER:**

10% dextran sulphate  
6X SSC  
1% SDS  
100 $\mu$ g/ml denatured salmon sperm DNA

**FREEZING MEDIA:**

10% DMSO

50% Heat Inactivated Newborn Calf Serum



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## SHORT REPORT

**A study of stabilisation of p53 protein versus point mutation in colorectal carcinoma**K.J. Cripps, C.A. Purdie<sup>1</sup>, P.J. Carder<sup>2</sup>, S. White, K. Komine, C.C. Bird & A.H. Wyllie

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Abnormalities of the *p53* tumour suppressor gene occur in many types of cancer including approximately 60% of colorectal carcinomas. This study investigates in 47 colorectal carcinomas the relationship between stabilised *p53* protein detected by immunocytochemistry (ICC), and *p53* mutation. 27 cases stained positively with the antibody PAb1801. Sequencing of exons 5–8 revealed 19 mutations in 18 of these cases (one tumour contained two different mutations). A rapid, non-radioactive method was developed to screen for mutations in this region of the gene involving Single Strand Conformational Polymorphism analysis (SSCP) and a *MspI* restriction digestion. This screen detected 17/19 (89%) of the sequenced mutations, and a further four mutations in 20 PAb1801 negative cases that were confirmed by sequencing. Reproducibility of ICC in detecting stabilised protein was assessed by restaining the 47 cases with the antibody DO7 after pre-treatment to optimise detection. Fewer cases were negative with DO7 although overall concordance with PAb1801 was good. A substantial proportion of carcinomas with stabilised *p53* as detected by ICC do not contain mutations in exons 5–8, whilst some mutations (the majority in exon 6) are not associated with stabilisation.

Abnormalities of the *p53* tumour suppressor gene are amongst the commonest genetic alterations recorded in human neoplasia. Mutations in *p53* occur in primary cancers in many sites including lung, breast, brain and colorectum (Nigro *et al.*, 1989). Greater than 60% of colorectal carcinomas show allelic deletion of the region 17p13 containing the *p53* gene and the remaining allele often contains a point mutation, usually arising at about the time of adenoma to carcinoma conversion (Vogelstein *et al.*, 1988; Baker *et al.*, 1989). 90% of point mutations tend to cluster in one of five evolutionary conserved regions and are commonly missense mutations resulting in a protein with an altered conformation (Nigro *et al.*, 1989; Gannon *et al.*, 1990; Soussi *et al.*, 1990; Hollstein *et al.*, 1991). Many mutations alter the biochemical properties of *p53*, including its ability to bind DNA, and act as a transcriptional transactivator (Raycroft *et al.*, 1990; Kern *et al.*, 1991).

In normal cells, *p53* is expressed at a low level and

has a short half-life (6 min in the spleen; Rogel *et al.*, 1985). A change in molecular configuration leads to stabilisation of the protein product so that its half life is greatly extended, thereby allowing detection by immunocytochemistry (ICC) (Jenkins *et al.*, 1985). Many of the tumour-associated missense mutations in *p53* cause protein stabilisation and it has often been assumed that stabilised *p53* visualised by ICC is equivalent to mutated *p53*. Recently however, it has become clear that the association between stabilisation and mutation is far from absolute (Wynford-Thomas, 1992). This may reflect both the type of mutation and the nature of the cells in which it occurred. A study of *p53* in 33 lung cancer cell lines revealed a correlation between missense mutations of the gene and strong overexpression of the protein (Bodner *et al.*, 1992), but, not all *p53* mutations result in a stabilised protein product. For example, Li–Fraumeni patients carry germline *p53* mutations yet mutant protein is detectable in only a proportion of cases (Frebouret *et al.*, 1992). Conversely not all stabilised protein is mutated. Wild type *p53* protein levels rise after DNA damage as the natural response to agents such as UV irradiation and the protein assumes a stable configuration detectable by ICC (Hall *et al.*, 1993), whilst some cancer family patients express stabilised unmutated *p53* protein in a substantial proportion of their cells (Barnes *et al.*, 1992). An enzyme-linked immunoassay has recently shown that *p53* stabilisation occurs in some colorectal adenomas in the absence of mutation (Tominga *et al.*, 1993). A study of *p53* staining in breast cancers revealed cytoplasmic staining of wild-type *p53* protein in 37% of cases, suggesting that nuclear exclusion may be a further mechanism in the inactivation of *p53* tumour suppressor activity in some tumours (Moll *et al.*, 1992). Finally there is evidence to suggest that stabilised wild type *p53* protein can arise due to the activation of other genes already implicated in cancer, such as *c-myc* and *ras* (Lu *et al.*, 1992; Wynford-Thomas, 1992). Recently it has been suggested that the tumour cell environment may impose a stable configuration on wild type protein, perhaps causing the loss of its normal growth regulatory responses (Vojtesek & Lane, 1993).

In this paper we have attempted to determine the frequency with which mutation and stabilisation occur in colorectal tumours. There are particular reasons for studying *p53* in colorectal cancer in this way. Firstly it has been shown that the presence of stabilised *p53* correlates with a particular class of cancers in which there are multiple divergent, near-tetraploid tumour cell clones and the suggestion has been made that such abnormal *p53* may facilitate endoreduplicative events leading to aneuploidy (Carder *et al.*, 1993). Secondly,

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Received 1 February 1994; accepted in revised form 18 May 1994

there are interesting comparisons to be made between primary cancers of the colorectum and at other sites. Existing evidence suggests that p53 can be involved in carcinogenesis in different ways. Thus, both colorectal and breast cancer show frequent stabilisation, but point mutation frequency in breast cancer is relatively low, commonly 20–30% (Bártek *et al.*, 1991; Dunn *et al.*, 1993) (although a recent paper by Jacquemier *et al.* (1994) indicates that the correlation between stabilisation and mutation of p53 in breast cancers may be higher than the previous studies suggest) whilst in existing series of colorectal cases, the incidence of p53 point mutations is greater than 50% (Nigro *et al.*, 1989; Rodrigues *et al.*, 1990). Overexpression of p53 protein without mutation in non-Hodgkin's lymphomas has also been demonstrated (Villuendas *et al.*, 1993). Thirdly, p53 ICC on colorectal tumours as in other cancers shows a variety of staining patterns. In some, staining is intense and affects the majority of nuclei within the section, in others it is widespread but involves fewer nuclei, and in some there is only focal staining that may affect a very low proportion of nuclei (Vojtesek *et al.*, 1993). Rather little is known of the biological meaning of these different patterns.

Accordingly we studied 47 colorectal carcinomas originally classified as positive or negative dependent on staining with the monoclonal antibody PAb1801 (Purdie *et al.*, 1991; Carder *et al.*, 1993). Mutations were defined in ICC positive cases by sequencing through exons 5–8, the region in which over 90% of all p53 mutations have been shown to occur in previous studies (Levine *et al.*, 1991). We then validated a method for screening for mutations using PCR-SSCP and an MspI restriction enzyme digest in ICC positive cases and applied it to ICC negative cases. Finally, the 47 cases were re-examined with a second antibody, DO7, under conditions which were more sensitive in detecting stabilised p53. Mutations outside exons 5–8 were searched for in the few cases in

which strongly positive staining was not associated with mutations within this region. The combined data support a strong relationship between stability and mutation, but highlight specific situations where stability and mutation are not concordant.

### Mutation analysis of PAb1801 positive tumours

Paraffin sections of 47 colorectal carcinomas fixed in PLPD, were stained with the antibody PAb1801 as previously described (Purdie *et al.*, 1991). In this analysis any nuclear reaction product in a section was deemed sufficient to classify the tumour as ICC positive and so included all three staining patterns described above. Positive staining was observed in 27 of the tumours. A total of 19 mutations in 18 (67%) of these cases were found by sequencing exons 5–8 (Table 1). (One case T4, contained two different mutations, CGC→CAC in codon 175 and CGG→CAG in codon 248.) The majority of mutations (12/19) clustered within two recognised hotspots at codons 175 and 248 (Hollstein *et al.*, 1991). One case (T8) was found at an exon/intron boundary (G→A of the first base of intron 5), and another (T12) was a conservative base change at codon 227. This last mutation is unlikely to be responsible for the stabilisation of the protein, which must therefore be attributable either to a second mutational event outside the region studied or to a non-mutational mechanism. Of 19 mutations 17 were either C→T or G→A transitions, 13 occurring at CpG dinucleotides. The remaining two cases were a G→T and a T→G transversion. The nature of these mutations thus concurs with the published literature in which the majority of p53 mutations in colorectal cancer are transitions at CpG dinucleotides within recognised hotspots (Hollstein *et al.*, 1991). Of the 27 tumours with stabilised p53, nine (33%) did not contain a

**Table 1** A comparison between location of mutation in 23 tumours and immunocytochemistry with PAb1801

Case	Immunocytochemistry positive		Immunocytochemistry negative		Amino acid change
	SSCP positive	SSCP negative	SSCP positive	SSCP negative	
T1	Exon 5	codon 152 CCG-CTG			Pro-Leu
T2		codon 175 CGC-CAC			Arg-His
T3		codon 175 CGC-CAC			Arg-His
*T4		codon 175 CGC-CAC			Arg-His
T5			codon 175 CGC-CTC		Arg-Leu
T6		codon 179 CAT-TAT			His-Tyr
T7		codon 180 GAG-TAG			Glu-stop
T8				exon/intron 5 Ggt-Gat	
T9	Exon 6		codon 202 CGT-CAT		Arg-His
T10			codon 203 GTG-ATG		Val-Met
T11			codon 213 CGA-TGA		Arg-stop
T12	Exon 7			codon 227 TGCT-TCG	Ser-Ser
T13		codon 245 GGC-AGC			Gly-Ser
T14		codon 248 CGG-TGG			Arg-Trp
T15		codon 248 CGG-TGG			Arg-Trp
T16		codon 248 CGG-TGG			Arg-Trp
T17		codon 248 CGG-TGG			Arg-Trp
T18		codon 248 CGG-TGG			Arg-Trp
*T4			codon 248 CGG-CAG		Arg-Gln
T19			codon 248 CGG-CAG		Arg-Gln
T20			codon 248 CGG-CAG		Arg-Gln
T21			codon 248 CGG-CAG		Arg-Gln
T22	Exon 8	codon 281 GAC-AAC			Arg-Asn

All individual tumours in which a mutation was detected by sequencing except \* in which the two mutations were detected in the same tumour. In addition T23, a tumour negative for immunocytochemical staining and for SSCP in exons 5–8 contained a TTC-TCC (Phe-Ser) mutation at codon 113 of exon 4



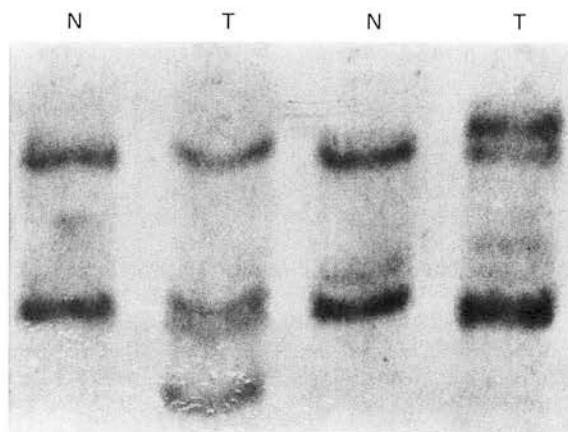
mutation within exons 5–8. In order to determine whether this was due to mutations outwith exons 5–8, the entire coding region was sequenced in three of these cases showing strong staining of the majority of nuclei with both antibodies (data not shown). A mutation in exon 4 was detected in one of the tumours resulting in a phenylalanine to serine amino acid change at codon 113. Exons 4 and 9 were then sequenced in the remaining six cases but no further mutations were found. Therefore 8/27 (30%) cases with positive immunocytochemistry appear to have no mutation suggesting stabilisation of p53 by other means.

#### PCR-SSCP/MspI screening for mutations in PAb1801 positive tumours

Rather than search for mutations in all the PAb1801 negative cases by sequencing, we sought to develop a more rapid but accurate screening method based upon SSCP of exons 5–8. This method derived from that of Orita *et al.* (1989) but did not use radioactive isotope. To validate the method we first applied it to the PAb1801 positive tumours where the sequence within these exons was known. 13/19 (68%) of the sequence-detected mutations revealed an abnormal band migration pattern under standard SSCP conditions (room temperature and 5% glycerol; Figure 1). Six mutations were not detectable as an SSCP. One of these was the exon/intron boundary mutation which would have been masked by the PCR primers used in the SSCP method. The conservative base change at codon 227 was also undetectable as an SSCP. The remaining four cases were all codon 248 CCG→CAG mutations. These cases presumably represent nucleotide changes insufficient to alter the secondary conformation to a level detectable by SSCP. SSCP was repeated under different conditions (4°C with a glycerol free gel), but no further mutations were detected. In order to extend this screening method to include reliable detection of the common codon 248 mutations, a PCR fragment containing exons 7–9 (primers 890 and 891; Figure 2) was digested with the restriction enzyme MspI. Disruption of the CCGG MspI recognition site at codon 248 by mutation resulted in the loss of 135 bp and 168 bp bands and addition of a 303 bp band (Figure 2). The combination of SSCP and MspI digestion detected 89% of mutations known to exist in exons 5–8 on the basis of sequencing. Moreover, all SSCP abnormalities were confirmed as mutations (no false positives). This method of screening for p53 mutations has the further advantage of being non-isotopic and substantially faster than sequencing.

#### Screening for mutations in PAb1801 negative tumours

Of the 47 tumours studied, 20 were classified as ICC negative. In these, no PAb1801 staining was seen within the section. Nonetheless four (20%) of these ICC negative tumours had mutations detected by the screening method (all showed SSCP abnormalities) and confirmed by sequencing (see Table 1). One of the mutations was at codon 175 within exon 5, whilst the remaining three were all in exon 6. In contrast, no exon

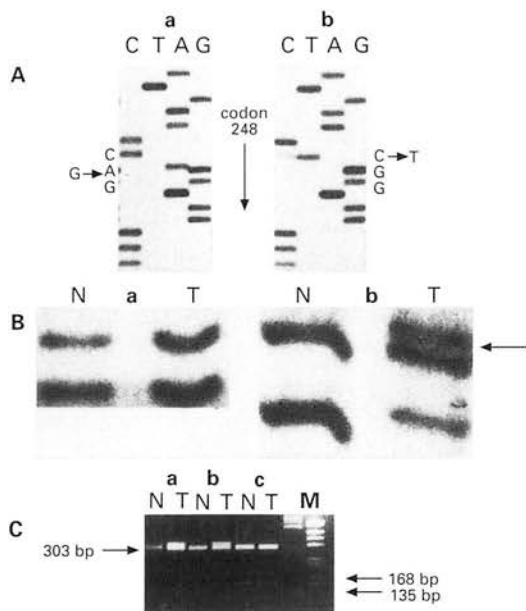


**Figure 1** Single Strand Conformational Polymorphism gel of exon 6 from two cases (T10 and T11) showing extra bands in the tumour samples as compared to the corresponding normals. (N-normal, T-tumour.) The polymerase chain reaction was performed on a 0.1–1 µg paired normal and tumour genomic DNA samples, in a 100 µl reaction containing 200 µM of each dioxynucleotide, 50 pmoles of each primer, and 2 units of a thermostable Taq polymerase in the relevant buffer. PCR was performed in a DNA thermocycler (Hybaid) with the following temperature profile: 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and 1 cycle of 72°C for 10 min. The primers were as follows (5' to 3'): exon 5 up-TTCCTCTTCTACAGTAGTC and 5 reverse-CGATGGT-GAGCAGCTGGG, exon 6 up-CCTCACTGATTGCTCTTA-GG and 6 reverse-CTGAGGTCTGGTTTGCAACT, exon 7 up-TGTGTTATCTCCTAGGTTGG and 7 reverse-GTCAGGAGC-CACCTTGCCA, exon 8 up-TCCTATCCTGAGTAGTGGT and 8 reverse-CGAGGTAAGCAAGCAGGA. The samples were extracted once with 24:1 chloroform/isomylalcohol to remove any mineral oil. 5–10 µl were then denatured in 80 µM NaOH, 10 µM EDTA at 48°C for 5 min, 2 µl sequencing stop solution was added, and the whole sample loaded onto a 5% glycerol, 0.5× MDE Hydronlink gel (Hoefer Scientific). The gel was run in 1×TBE on the SE600 PAGE apparatus (Hoefer Scientific) at 25°C, 20W for 2–3 h (alternatively alternative conditions of 4°C in the absence of glycerol were used in some cases). Bands were visualised by a silver stain (BioRad) as per manufacturers instructions. The gel was dried onto 3MM paper and laminated.

6 mutations were found amongst the PAb1801 positive cases.

#### DO-7 Immunocytochemistry

It is possible that failure to identify stabilised product in the presence of mutated p53 merely reflected the sensitivity of the method of staining. To address this, we restained the sections under optimised conditions of microwave pre-treatment with the human p53 specific murine monoclonal antibody DO-7 (Cattoretti *et al.*, 1993). The two antibodies showed similar patterns of staining with the three classes of staining pattern discussed above readily discernible. Although microwave pre-treatment and DO-7 staining produced fewer negative cases than PAb1801 (nine vs 20), the four PAb1801 negative cases with mutation were also all clearly negative with DO-7. The epitopes recognised by PAb1801 and DO-7 are both within the N-terminal domain of p53 (Banks *et al.*, 1986; Vojtěšek *et al.*, 1992) and it was therefore possible that both antibodies might fail to detect a genuinely stabilised protein through some epitope masking phenomenon. To exclude this, the three cases with exon 6 mutations that were negative with both antibodies were stained



**Figure 2** (A) Sequencing gel of exon 7 from two cases showing the two different codon 248 mutations (a) (T19) CCG-CAG and (b) (T14) CGG-TGG. (B) Single Strand Conformational Polymorphism gel of the two cases above showing an abnormality (indicated by arrow) in (b) but not (a). (C) 3% agarose gel of an MspI restriction digest of exons 7–9 in both normal and tumour samples from cases (a), (b) and a non-mutated control case (c). The mutations at codon 248 are seen as a loss of the 135 and 168 base pair bands due to mutation of a MspI site resulting in a 303 base pair band, in addition to the constant 276 and 290 base pair bands. (The presence of the 135 bp and 168 bp bands in tumour sample a.) is due to contaminating normal tissue). (N-normal, T-tumour, M-DNA molecular weight marker.) Sequencing was performed by one of three methods using the PCR primers as sequencing primers: (1) Cycle sequencing of PCR amplified exons 5–8. Briefly, the components of a chain termination reaction were cycled through a temperature profile of heat denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min in the presence of  $\gamma$ -<sup>32</sup>P-labelled primer and 50 fmol of PCR product (after purification with Promega Magic Clean-up Columns). (2) Subcloning of a PCR product containing exons 7–9 using primers 890 and 891 into pUC18, pooling of approx. 100 transformed colonies, and miniprep DNA preparation using Magic Mini-prep kit (Promega) followed by the chain termination method with [ $\alpha$ -<sup>32</sup>S] as per the manufacturers protocol for dsDNA (United States Biochemicals, Sequenase Version 2.0) 890-5'-GAGAGAAGCTTGCCACAGGTCCTCCCAAGGCGA-A; 891-5'-AGAGAGGGATCCACTTTCCACTTGATAAGAG-GTCCCAAG. (3) Separation of the two strands by Dynabeads (Dyna) after PCR amplification of exons 5–6 using a biotinylated upstream primer and the exon 6 reverse primer (see PCR-SSCP), followed by the chain termination method as above. Biotinylated primer as follows (5'-3') AGGAGGTGCTTACA-CATGTT. Samples were then denatured at 95°C for 5 min, electrophoresed through a 6% urea/acrylamide gel, fixed for 10 min in a 10% methanol/10% acetic acid solution, dried on 3MM paper, and exposed to autoradiographic film overnight.

with the polyclonal antibody CM-1 and no positive staining was seen although CM-1 convincingly detected stabilised protein in three further known DO-7 positive cases included in the same run (data not shown). Finally, no cases with single scattered positively staining nuclei with either PAb1801 or DO-7 contained mutations, but mutations were found in two cases with only 30–50% moderately strongly positive nuclei on staining with DO-7. As mentioned earlier, two tumours in which the majority of nuclei stained strongly with both antibodies failed to reveal mutations in the entire coding region of the gene.

## Discussion

The data clearly show that positive ICC staining with either of the antibodies studied does not correlate exactly with *p53* mutation. It is difficult to give absolute figures for this disparity as the proportion of cells staining differs with the method used and the percentage of positive cases is also dependent upon the extent to which tumours with sparsely or focally distributed positive nuclei are included. This study shows however that mutations can be found in approximately 20% of colorectal carcinomas that are ICC negative, and that mutation in exons 5–8 cannot be found in as many as 33% of ICC positive colorectal cancers. The group of ICC positive cancers without apparent *p53* mutation in exons 5–8 contains all the cases in which stabilised *p53* protein was evident only in rare widely-dispersed nuclei. However there were also three cases that showed the more familiar staining pattern, with the majority of nuclei strongly positive. One of these was shown to contain an exon 4 mutation at codon 113. Occasional mutations in both exons 4 and 9 have been reported in the literature and although this was the only mutation recorded outwith exons 5–8 in this study, these observations suggest that previous studies restricted to exons 5–8 may have slightly underrepresented the true incidence of *p53* mutations in colorectal tumours. Amongst the mutated cases that were ICC negative we noted a predominance of mutations in exon 6. It is probable that these tumours contained only mutant protein as two of the three cases showed loss of alleles within *p53* in the tumour whilst the remaining case was non-informative with a variety of polymorphic probes (data not shown). Previous studies of ICC and mutation in colorectal and ovarian cancers also show that exon 6 mutations often fail to result in a stabilised protein (Cunningham *et al.*, 1992; Kikuchi-Yanoshita *et al.*, 1992; Teneriello *et al.*, 1993). Taken together these results suggest that exon 6 includes a region of the gene in which mutations frequently fail to stabilise *p53*, whilst still conferring a selective growth advantage. The presence of mutations in occasional ICC negative tumours and the absence of mutations in some strongly ICC positive tumours suggests that alternative mechanisms of *p53* abnormality (stabilisation, mutation or both) occur relatively frequently in colorectal cancers, a tumour type in which identity between stabilisation and mutation has often been assumed. An absence of direct correlation between stabilisation and mutation has been reported in other tumour types including breast carcinoma and non-Hodgkin's lymphoma (Dunn *et al.*, 1993; Villuendas *et al.*, 1993). As larger case numbers accrue in which the distinction is made between mutational and non-mutational mechanisms of stabilisation, it will be possible to determine whether this distinction is accompanied by differences in tumour behaviour. This paper provides details of a non-radioactive method by which almost all *p53* mutations can be rapidly and accurately identified, so facilitating such studies.

## Acknowledgements

We thank P. Chumakov and J. Jenkins for primer sequences 890 & 891, S. Gledhill for assistance with primer design, D. Bishop and staff for help with the

immunocytochemistry and D.P. Lane for the antibodies PAB1801 and DO7.

KJC holds a Cancer Research Campaign Studentship.

CAP was supported by a University of Edinburgh Medical Faculty Fellowship. SW was supported by a grant from SHHD.

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## Letter

0959-8049(94)00517-6

### Mutational Analysis of the MCC Gene by Single-strand Conformational Polymorphism Analysis

K.J. Cripps, L.J. Curtis and A.H. Wyllie

A REGION on chromosome 5q21 is commonly deleted in sporadic colorectal carcinomas, and has been shown to contain the gene responsible for familial polyposis coli (FPC), an inherited form of colorectal cancer [1, 2]. Several genes have been mapped to this region including both *APC* and *MCC* [3-6]. *APC* has since been shown to be the gene responsible for FPC, and is also mutated somatically in the majority of sporadic colon cancers. The role of *MCC* is, however, still uncertain. No mutations of *MCC* have been found in FPC patients, and only six somatic mutations in sporadic tumours have been reported [6, 7].

This study screened the 17 exons of the coding region of the *MCC* gene for mutations in a small subset of clinically important colorectal tumours from five non-polyposis patients who presented with the disease at a relatively young age (Table 1). We considered it possible that defects in *MCC* might be important in colorectal tumorigenesis in this subset of younger non-polyposis patients whilst having little input on the development of sporadic tumours in older patients. Mutational analysis was performed by the technique of single-strand conformational polymorphism analysis after amplification by the polymerase chain reaction (PCR-SSCP) on normal and tumour tissue as described previously [6] using PCR primers previously published [8]. We used DNA from these tumours previously grown as xenografts in SCID mice to eliminate the presence of any normal stromal elements which may have rendered mutational analysis difficult.

The method detected abnormalities in all 5 previously reported cases with the *MCC* mutation. No mutations, however, were found in the coding region of *MCC* in any of the 5 young patients, although several polymorphisms were noted. A deletion polymorphism in exon 10 and a C-T conservative base change at codon 708 of exon 15 have been reported previously [9]. A novel polymorphism was observed in two of the 5 patients. Sequencing of these samples revealed a two base pair change (GC-TG) within intron 13 immediately prior to exon 14. This polymorphism appeared to be linked to the polymorphism in exon 15 and this was confirmed by extending the study of these two exons to an additional 19 constitutional DNA samples from young non-polyposis colorectal cancer patients and 18 normal control patients. The allele frequency of the novel polymorphism (B1 in Table 1) was 83% compared to the published sequence of the gene. No difference in frequency was observed between the normal and young colon cancer populations.

A recent study from this laboratory showed that whilst allele loss on 5q21 invariably involved both *MCC* and *APC*, no mutations of *MCC* could be detected by PCR-SSCP in the remaining copy of the gene [10]. These combined studies suggest that *MCC* does not have a role as an independent tumour suppressor gene in colorectal cancer, at least at the level studied.

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**Acknowledgement**—We wish to thank K. Kinzler for communicating the exon 19 polymorphism and supplying the positive control samples.

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Received 7 Nov. 1994; accepted 24 Nov. 1994.



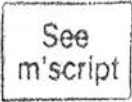


Table 1. Clinico-pathological and molecular genetic data

Patient number	Sex and age (years)	Family history of colorectal disease	Location of tumour	Dukes stage	5q21 status*	MCC exon 10	MCC exon 14	MCC exon 15	Tp53 ICC	17p status†	Tp53 mutation	Ki-ras (codon 12)‡
1	Male 48	—	Ascending colon	C	Loss	A1/A1*	B1/B1	C1/C1	+	Loss	cd248	Mutated
2	Male 40	—	Sigmoid colon	C	Loss	A2/A2	B2/B2	C2/C2	—	Non-informative	cd175	Wild-type
3	Male 45	Brother	Sigmoid colon	C	Retained	A1/A2	B1/B1	C1/C1	+	Retained	cd248	Wild-type
4	Male 39	Maternal aunt	Sigmoid colon	A	Loss	A1/A1	B1/B1	C1/C1	—	non-informative	nd	Wild-type
5	Male 36	Father, two brothers, grandmother and aunt	Unknown‡	D	Retained¶	A1/A1	B1/B1	C1/C1	—	Retained	nd	nd

\* Previously identified by a variety of polymorphic probes flanking APC and MCC.  
† Established from LOH at YNZ.22 or polymorphic loci within Tp53.  
‡ Established by sequencing.  
§ Peritoneal carcinomatosis at operation.  
¶ Retained at the one site informative within the APC gene.  
‡ A1/B1/C1 refers to the more frequent allele in the population, whilst A2/B2/C2 refers to the less frequent polymorphic allele.  
nd, not done.



# Mutation of the p53 gene precedes aneuploid clonal divergence in colorectal carcinoma

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**Summary** To establish whether p53 mutation precedes or follows clonal divergence in human colorectal carcinomas, 17 tumours were analysed at multiple sites (2–5 each) for single-strand conformation polymorphisms (SSCP) within exons 5–8 of the p53 gene. A previous study had demonstrated subclones of differing DNA ploidy in these tumours, but all showed immunocytochemical evidence for p53 stabilisation, using the monoclonal antibody PAb 1801. Mutations within exons 5–8 of p53 were identified by the presence of an abnormally migrating band in 10 of the 17 carcinomas: five in exon 5, four in exon 7 and one in exon 8. In each of these positive cases, samples from different parts of the carcinoma showed identical gel migration patterns in SSCP analysis. Similarly, the remaining seven tumours were concordant for absence of band shift across all samples of each tumour. Six SSCP-positive cases contained multiple populations differing in DNA ploidy, while four were homogeneously diploid or aneuploid throughout. Very similar proportions were observed in the SSCP-negative cases. In four positive tumours the mutation was confirmed by sequencing or through alteration of nucleotide-specific restriction enzyme cleavage. Identical mutations appeared in every sample from the same tumour. The results provide unequivocal evidence that the same mutant allele of p53 is present throughout each tumour bearing a mutation, regardless of the clonal variation identified by analysis of DNA ploidy. We conclude that in colorectal tumorigenesis mutation of p53 occurs as a single event which precedes and may facilitate the aneuploid clonal divergence of carcinomas.

**Keywords:** colorectal carcinoma; p53, SSCP; clonal evolution

Inactivation of the tumour-suppressor gene p53 is an important step in the development of the majority of human cancers (Hollstein *et al.*, 1991). Functional inactivation occurs most commonly as a result of missense mutation (Baker *et al.*, 1989) but can also result from interaction with oncogenic viral or cellular proteins (Mietz *et al.*, 1992; Momand *et al.*, 1992). The p53 protein is a sequence-specific DNA-binding protein that is active as a transcription factor (Bargonetti *et al.*, 1991) and can interact directly with the replication apparatus (Dutta *et al.*, 1993). There is good evidence that its normal function is to establish G<sub>1</sub> checkpoint control in response to DNA damage, so allowing time for DNA repair (Kastan *et al.*, 1991) or the initiation of apoptosis (Clarke *et al.*, 1993). Lack of functional p53 promotes genomic instability (Bischoff *et al.*, 1990; Harvey *et al.*, 1993), which is probably a key factor in acquisition of the multiple 'hits' required for carcinogenesis (Nowell, 1976).

We have recently shown that human colorectal carcinomas containing immunohistochemically detectable p53 are more likely to contain multiple aneuploid DNA stem lines than those which are p53 negative (Carder *et al.*, 1993). This result is in keeping with a relationship between p53 and genomic instability. We also reported that in carcinomas containing stabilised p53 the abnormality was almost always present throughout the tumour even though DNA analysis revealed the presence of divergent stem lines. From these findings we concluded that p53 stabilisation is a critical early event in cancer evolution favouring the development of tetraploid and other aneuploid sublines. Since it is now clear that p53 stabilisation can be the result of the tumour cell environment, independent of mutation (Vojtesek and Lane, 1993), we felt it was important to re-examine these cases for more definitive evidence, firstly, that mutation of p53 had occurred and, secondly, that it preceded clonal divergence in tumour progression. To this end we used the technique of single-stranded conformation polymorphism (SSCP) analysis (Orita *et al.*, 1989; Glavac and Dean, 1993) to identify mutant alleles of p53 and confirm that the same mutant allele was present in all of the samples taken from any one carcinoma, despite independent evidence of clonal divergence between these samples.

## Materials and methods

### Samples

Sixty-four samples from 17 colorectal carcinomas from our previously published series (Carder *et al.*, 1993) were studied. An average of four samples per tumour (range 2–5) were analysed and compared with normal colonic mucosa from the same individual. All samples of all tumours contained stabilised p53 as determined by immunohistochemistry using PAb 1801 (Oncogene Science). Flow cytometry was performed on frozen tissue and immunohistochemistry was performed on tissue fixed in periodate lysine paraformaldehyde (PLPD), both as described previously. To minimise possible confusion between tumour and non-neoplastic stroma, samples were assessed as diploid only if tumour cells occupied in excess of 50% of tissue sections from which the corresponding flow cytometric analysis showed a diploid main peak. Samples were considered aneuploid if a separate peak, distinct from the diploid peak, was identified, and aneuploid populations from different samples of the same tumour were considered identical unless they differed in DNA index by more than 0.1. Samples were considered tetraploid if a separate peak with DNA index between 1.9 and 2.1 comprised more than 10% of the nuclei.

### Single-strand conformation polymorphism (PCR-SSCP) analysis

The polymerase chain reaction (PCR) was performed on 0.1–1 µg of genomic DNA samples, in an 100 µl reaction containing 200 µM of each deoxynucleotide, 50 pmol of each primer and 2 units of a thermostable *Taq* polymerase in the relevant buffer. PCR was performed in a DNA thermocycler (Hybaid) with the following temperature profile: one cycle of 94°C for 5 min, 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and one cycle of 72°C for 10 min. The primers were as follows (5' to 3'): exon 5 up TTCCTCTTC-CTACAGTAGTC and 5 reverse CGATGGTGAGCAGC-TGGG; exon 6 up CTCCTACTGATTGCTCTTAGG and 6 reverse CTGAGGTCTGGTTTGCAACT; exon 7 up TGTGTTATCTCCTAGGTTGG and 7 reverse GTCAG-GAGCCACTTGCCA; exon 8 up TCCTATCCTGAGTAG-TGGT and 8 reverse CGAGGTAAGCAAGCAGGA. The

samples were extracted once with 24:1 chloroform/isoamylalcohol to remove any mineral oil and 5–10 µl was then denatured in 80 µM sodium hydroxide, 10 µM EDTA, at 48°C for 5 min. Two microlitres sequencing stop solution was added, and the whole sample loaded onto a 5% glycerol, 0.5 × MDE Hydrolink gel (Hoefer Scientific). The gel was run in 1 × TBE on the SE400 PAGE apparatus (Hoefer Scientific) at 25°C, 20 W, for 2–3 h.

Normal and tumour samples from the same individual were run together for comparison. Tumour samples were classified as SSCP positive if a discrete additional band was observed. Bands were visualised by a silver stain (BioRad) as per the manufacturer's instructions with additional washes. The gel was dried on to 3MM paper and laminated.

#### Sequence analysis

A total of nine samples from two cases with band shift on SSCP throughout were sequenced by the dideoxy chain-termination technique as described previously (Cripps *et al.*, 1994) using a commercial kit (Sequenase, Amersham).

#### Restriction enzyme digestion using *MspI*

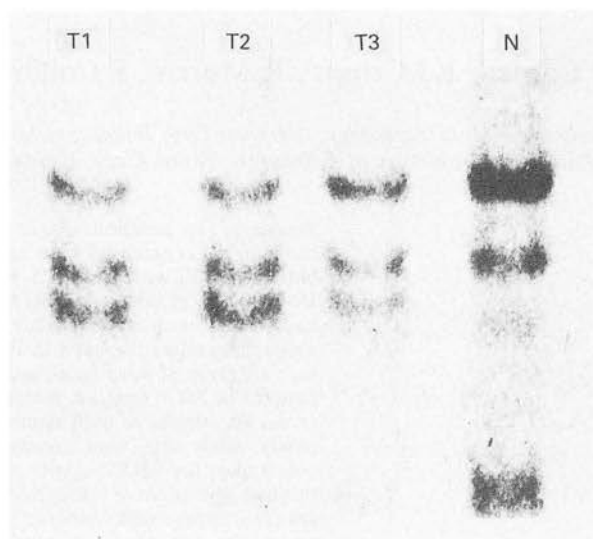
A total of eight samples from two cases with band shift on SSCP throughout and known to contain a CGG→CAG mutation in codon 248 were analysed by restriction enzyme digestion using *MspI*. As described previously (Cripps *et al.*, 1994), disruption of the CCGG recognition site by mutation results in loss of the 135 bp and 168 bp bands and creates an additional 303 bp band.

### Results

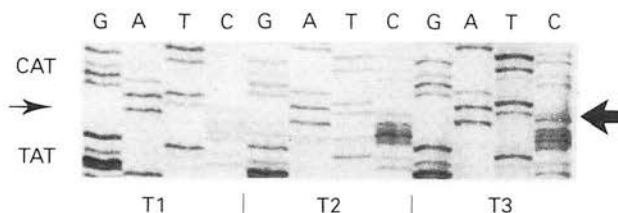
Seventeen carcinomas which had been sampled at multiple sites and demonstrated immunohistochemical positivity for PAb 1801, and for which DNA ploidy values were available, were studied. In 14 cases the majority of nuclei stained intensely. Three cases showed the 'mosaic' pattern, in which positive nuclei were scattered sparsely throughout the tumour. Twelve cases contained multiple clonally distinct subpopulations as determined by assessment of DNA ploidy by flow cytometry, with eight containing two and four containing three variant subpopulations.

From each tumour sample exons 5–8 of the p53 gene were amplified individually and analysed by SSCP. Band shifts indicating the presence of a mutant p53 allele with altered conformation were detected by SSCP in 10 of the 17 carcinomas (59%). An average of four samples per tumour were studied, and the same band shift was present in all samples of each SSCP-positive case, suggesting a single clonal mutation event (Figure 1). The presence of a mutation in the amplified p53 fragment revealing the band shift was confirmed in nine samples of two cases by direct nucleotide sequence analysis. Six samples from one case contained a CGC→CAC mutation in codon 175 and three samples from the other case contained a CAT→TAT mutation in codon 179 (Figure 2). In two further cases a mutation in codon 248 was confirmed in a total of eight samples using the *MspI* restriction enzyme digestion technique (Figure 3). In all, five mutations occurred in exon 5, four in exon 7 and one in exon 8. Six cases with SSCP-confirmed mutations contained multiple divergent populations as detected by assessment of DNA ploidy, indicating clonal evolution subsequent to mutation (Figure 4). All samples of the SSCP-negative cases were concordant for absence of band shift, confirming that mutation had not occurred (at any rate in this commonly affected part of the p53 gene) during carcinoma progression in these cases. All cases with mutation demonstrated strong positive staining for PAb 1801 in a majority of tumour cell nuclei, but four cases with similarly strong staining were SSCP negative in exons 5–8. The relationship between immunocytochemistry and mutation is described more fully

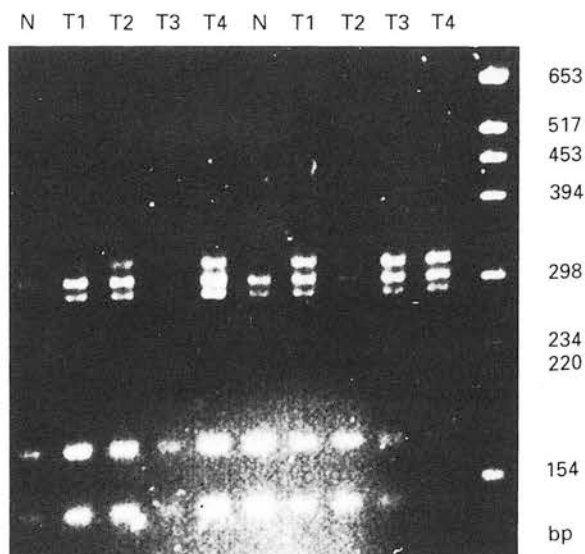
elsewhere (Cripps *et al.*, 1994), in a larger number of cases including the present 17.



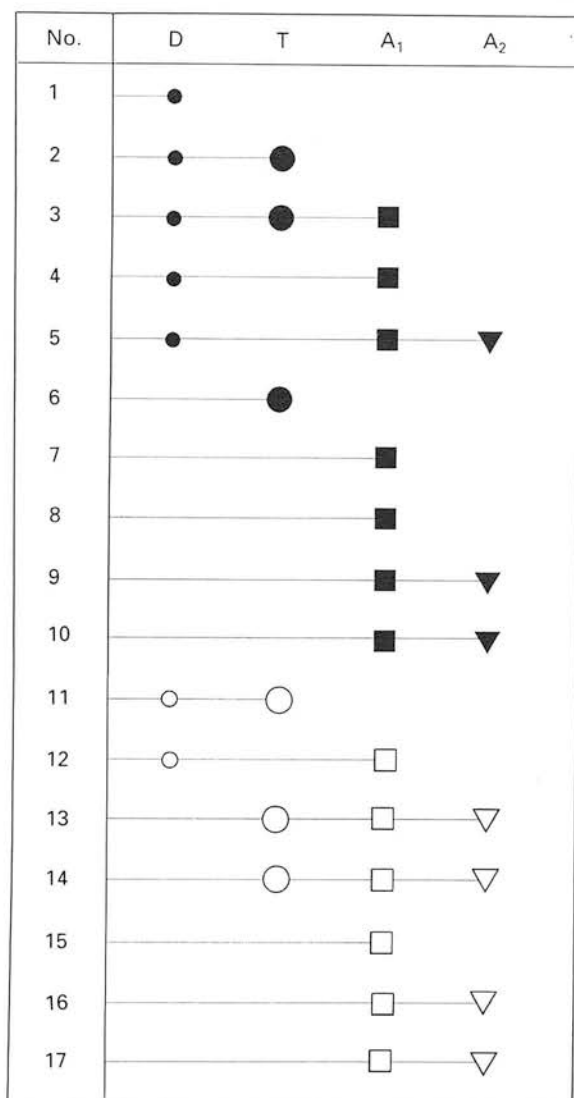
**Figure 1** Identical band shifts are revealed by SSCP analysis in all divergent subpopulations of a colorectal carcinoma. N, normal tissue; T1–3, multiple samples from the same carcinoma (DNA index: T1, 1.5/1.6; T2, 1.5; T3, 1.6).



**Figure 2** Dideoxynucleotide sequencing confirms a CAT→TAT mutation in codon 179 (exon 5) in all three samples from this tumour.



**Figure 3** *MspI* restriction digestion of exons 7–9 confirms a mutation in codon 248 in all tumour samples. Loss of the CCGG recognition site creates an additional 303 bp band and results in loss of the smaller 135 bp and 168 bp bands. The smaller bands are due to contaminating normal tissue.



**Figure 4** Relationship between p53 mutation and clonal evolution. Over all sampled stem lines from any one tumour, band shifts on SSCP analysis of exons 5–8 were either uniformly present (closed symbols) or uniformly absent (open symbols). o, diploid; O, tetraploid; other symbols aneuploid with different aneuploid stem lines within the same tumour being designated by different stem lines. D, diploid; T, tetraploid; A<sub>1</sub> and A<sub>2</sub>, aneuploid.

## Discussion

We have used the technique of single-stranded conformation polymorphism (SSCP) analysis to detect clonality of p53 mutation in colorectal carcinomas. SSCP provided a sensitive method of detecting mutations, as mutant alleles generate characteristic band shifts on electrophoresis under non-denaturing conditions (Orita *et al.*, 1989). The technique is sufficiently powerful to allow distinction between sequences differing in a single base pair (Cripps *et al.*, 1994). While in theory one might expect a mutant allele to create at least two additional conformers (one from each strand), often only one is observed, and it appears that most new conformers derive

from the purine-rich strand (Glavac and Dean, 1993).

Using SSCP we have demonstrated identical band shifts in the same exon in subclones of carcinomas that diverge in DNA ploidy, and also in carcinomas which are homogeneously diploid or aneuploid. We interpret this to indicate that p53 mutation occurs prior to divergence of clones differing in DNA ploidy, and hence any one tumour possesses a single p53 mutation throughout. In a large series of carcinomas reported separately (Cripps *et al.*, 1994) we have confirmed by sequencing that band shifts detected by SSCP invariably denote mutation, and that the technique used here identifies more than 70% of all naturally occurring mutations in the region of p53 studied (exons 5–8). It may be argued that identical band shifts might still represent different point mutations within the same amplified fragment. We feel that this interpretation is unlikely for three reasons. Firstly, even minor differences in nucleotide substitution (e.g. in adjacent positions of the same codon) can lead to profound differences in band shift by SSCP analysis using the methods employed here (Cripps *et al.*, 1994). Secondly, in the now considerable literature on p53 mutations in cancer, the occurrence of two distinct mutations in the same exon in any one tumour is uncommon. Finally, we have used direct sequencing to confirm identity of mutation throughout in two cases, and in a further two we have used a rapid restriction enzyme digestion technique to confirm identity of mutation site.

The observation that an identical mutation in p53 occurs throughout affected carcinomas, including those with and without clonal divergence in DNA ploidy, provides strong evidence for a single mutational event early in the development of these cancers. Around 20–30% of all colorectal carcinomas containing immunohistochemically stable p53 appear not to contain p53 mutations (Wynford-Thomas, 1993; Baas *et al.*, 1994; Cripps *et al.*, 1994) but examples of these in this series also showed divergent subclones concordant in p53 immunocytochemistry. Hence, even the non-mutational abnormalities that affect p53 stability may also be an early event. The data presented here emphasise the central role of wild-type p53 in preventing one type of genomic instability. This instability appears to result from continued DNA replication in the presence of DNA damage (Lane, 1992; Livingstone *et al.*, 1992; Yin *et al.*, 1993) and tends to produce near-tetraploid subclones (Carder *et al.*, 1993). It is interesting that the selective growth advantage afforded by this instability appears to be a feature favouring carcinoma rather than adenoma growth, since p53 mutations are unusual in adenomas. Other lesions involved in colorectal tumorigenesis but unrelated to p53, such as mutation in the DNA repair genes implicated in hereditary non-polyposis colon cancer (Peltomäki *et al.*, 1993), also initiate genomic instability, albeit of a different type, and associate preferentially with carcinoma rather than adenoma.

In conclusion we establish here by DNA analysis the impression gained from our previous immunohistochemical study: in colorectal tumorigenesis p53 mutation is a critical early lesion occurring as a single clonal mutational event which precedes and probably facilitates the emergence of divergent aneuploid tumour subpopulations.

## Acknowledgements

We would like to thank Juliet Hamblin for expert typing of the manuscript, Steven Toms for photography and Andrew Hay for the illustrations.

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